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<p>(54) Title: USE OF PAPILLOMAVIRUS E2 PROTEIN IN TREATING PAPILLOMAVIRUS-INFECTED CELLS AND COMPOSITIONS CONTAINING THE PROTEIN</p> <div style="text-align: center; margin: 10px 0;"> <p>ΔE1 (0.9-9.8mu)</p> </div> <p>(57) Abstract</p> <p>By virtue of the present invention, there are provided methods and compositions for interfering with the proliferation of cells infected and/or transformed by papillomaviruses. The processes and compositions of this invention may be used to treat any mammal, including humans. According to this invention, mammals are treated by the pharmaceutically acceptable administration of an E2_{ad/ds} protein, either directly or by gene transfer techniques, to reduce the symptoms of the specific papillomavirus-associated disease, or to prevent their recurrence.</p>		

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**USE OF PAPILLOMAVIRUS E2 PROTEIN IN TREATING PAPILLOMAVIRUS-INFECTED CELLS
AND COMPOSITIONS CONTAINING THE PROTEIN*****Background of the Invention***

5 Papillomaviruses (PV) have been linked to widespread, serious human diseases, especially carcinomas of the genital and oral mucosa. Tens of millions of women suffer from human papilloma virus (HPV) infection of the genital tract. Significant number of these women eventually develop cancer of the cervix. It has been estimated that perhaps
10 are associated with HPV. As many as 90% of all cervical cancer may be linked to HPV.

 Papillomaviruses also induce benign, dysplastic and malignant hyperproliferations of skin and mucosal epithelium (see, for example, Mansur and Androphy, (1993) *Biochim Biophys Acta* 1155:323-345; Pfister (1984) *Rev. Physiol. Biochem. Pharmacol.* 99:111-181; and Broker et al. (1986) *Cancer Cells* 4:17-36, for
15 reviews of the molecular, cellular, and clinical aspects of the papillomaviruses).

 HPV's are a heterogeneous group of DNA tumor viruses associated with hyperplastic (warts, condylomata), pre-malignant and malignant lesions (carcinomas) of squamous epithelium. Almost 70 HPV types have been identified, and different papillomavirus types are known to cause distinct diseases, c.f., zur Hausen, (1991)
20 *Virology* 184:9-13; Pfister, (1987) *Adv. Cancer Res.*, 48:113-147; and Syrjanen, (1984) *Obstet. Gynecol. Survey* 39:252-265. HPV's can be further classified either high risk (such as HPV type 16 [HPV-16] and HPV-18) or low risk (or HPV-6 and HPV-11) on the basis of the clinical lesions with which they are associated and the relative propensity for these lesions to progress to cancer. For example, HPV types 1 and 2
25 cause common warts, and types 6 and 11 cause warts of the external genitalia, anus and cervix. HPV's can be isolated from the majority of cervical cancers, e.g., approximately 85 to 90% of human cervical cancers harbor the DNA of a high-risk HPV. Types 16, 18, 31 and 33 are particularly common; HPV-16 is present in about 50 percent of all cervical cancers.

30 The biological life cycle of the papillomaviruses appears to differ from most other viral pathogens. These viruses are believed to infect the basal or germ cells of the epithelium. Rather than proceeding to a lytic infection in which viral replication kills the cell, viral DNA transcription and replication are maintained at very low levels until higher strata of the epithelium are achieved. There, presumably in response to
35 differentiation-specific signals, viral transcription accelerates, DNA synthesis begins and virions assemble. It is within this terminally differentiating cellular environment that the virus must recruit the panoply of cellular factors necessary for its reproduction.

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In HPV-positive genital cancers, the viral genomes are transcriptionally active, and two viral genes, E6 and E7, are invariably expressed. The high-risk HPVs encode two oncoproteins, E6 and E7, whose expression can extend the life span of squamous epithelial cells, which are the normal host cells for the papillomavirus. E6 and E7 together can result in the efficient immortalization of primary human cells (Hawley-Nelson et al., (1989) *EMBO J.*, 8:3905-3910; Münger et al., (1989) *J. Virol.*, 63:4417-4421; Watanabe et al., (1989) *J. Virol.*, 63:965-969). E6 and E7 are expressed in HPV-positive cervical cancer-derived cell lines (Schneider-Gädick et al., (1986) *EMBO J.*, 5:2285-2292; Schwarz et al., (1985) *Nature*, (London) 314:111-114; Smotkin et al., (1986) *Proc. Natl. Acad. Sci. USA*, 83:4680-4684). Furthermore, although many genetic changes have occurred in cervical carcinoma cells, the continued expression of the viral oncoproteins is necessary since expression of antisense E6/E7 RNA results in decreased cell growth (von Knebel-Doeberitz et al., (1988) *Cancer Res.*, 48:3780-3785). Similar to the transforming proteins of the other small DNA tumor viruses, simian virus (SV40) and adenovirus, the transforming properties of the E6 and E7 oncoproteins appear to be due at least in part to their capacity to functionally inactivate the p53 and the retinoblastoma (pRB) tumor suppressor proteins. The E6 proteins of HPV-16 and HPV-18 can complex (Werness et al., (1990) *Science*, 248:76-79) and inactivate p53 (Mietz et al., (1992) *EMBO J.*, 11:5012-5020). The high-risk HPV E7 proteins bind pRB more efficiently than the E7 proteins of low-risk HPVs (Barbosa et al., (1990) *EMBO J.*, 9:153-160; Dyson et al., (1989) *Science*, 243:934-937; Münger et al., (1989) *EMBO J.*, 8:4099-4015). It is believed that the functional inactivation of the p53 and pRB regulatory pathways by E6 and E7 are important steps in cervical carcinogenesis.

One characteristic of HPV-related carcinogenic progression is the frequent integration of the viral genome into the human chromosome in the cancer cells in a manner that results in the loss of expression of the viral E2 gene but maintains high levels of E6/E7 expression (Dürst et al., (1985) *J. Gen. Virol.*, 66:1515-1522; Jeon et al., (1995) *Proc. Natl. Acad. Sci. USA*, 92:1654-1658). The product of the E2 open reading frame plays an important role in the complex transcriptional pattern of the HPV's. The E2 transcriptional activation protein ("the E2 protein") is a trans-acting factor that activates transcription through specific binding to cis-acting E2 enhancer sequences in viral DNA (Androphy et al., (1987) *Nature*, 324:70-73), and has been shown to induce promoter expression in a classical enhancer mechanism (Spalholz et al., (1985) *Cell* 42:183-91). The E2 gene product exerts trans-regulatory effects in the upstream regulatory region ("URR") of the viral genome and disruption of E2 is thought to alter regulation of expression of E6 and E7 genes. The upstream regulatory region is found

immediately 5' to the early genes of bovine papilloma viruses (BPV's) and other papillomaviruses. The URR contains cis-acting regulatory signals, including an origin of DNA replication and several promoters that function in early transcription. The URR also contains enhancer elements that activate transcription from the URR promoters and heterologous promoters (Sousa et al., (1990) *Biochemica et Biophysica Acta* 1032: 19-37).

The E2 enhancer elements are conditional, in that they stimulate transcription only when activated by a protein encoded by the E2 open reading frame. As with other transcription factors, the functions of E2 protein appear to be localized to discrete modular domains (Giri et al., (1988) *EMBO J.*, 7:2923-29). Gene products from the E2 gene include the full-length transcriptional activator E2 protein and at least two truncated versions of the E2 protein BPV1 that function as transcriptional repressors. Transcriptional activation and repression of viral genes by E2 gene products constitute critical regulatory circuits in papillomavirus gene expression and DNA replication.

Within the URR, transcriptional regulation by the E2 protein depends on its direct binding to the nucleotide sequence 5'ACC(G)NNNN(C)GGT3' (SEQ ID NO:9) (Androphy et al., *supra*; Dartmann et al., (1986) *Virology*, 151:124-30; Hirochika et al., (1987) *J. Virol.*, 61:2599-606 ; P. Hawley-Nelson et al., (1988) *EMBO J.*, 7:525-31; McBride et al., (1988) *EMBO J.*, 7:533-39). In that sequence, N represents any nucleotide; X is any nucleotide, but is usually G; and Y represents any nucleotide, but is usually C. E2 binding sites appear to be positioned in close proximity to the viral promoters, with multiple E2 binding sites present throughout the papillomavirus genome (R. Li et al., (1989) *Genes Dev.*, 3: 510-26), in the URR's of all papillomaviruses, as well as in other sites near promoters throughout the viral genome. Moreover, E2 binding sites may function as an element in viral DNA replication, as well as a classical transcriptional enhancer element.

The E2 protein also binds the papillomavirus replication protein known as E1. It has been proposed that when an E2/E1 complex binds to an E2 binding site, replication of the viral genome occurs (Mohr et al. *Science* (1990) 250:1654-99).

European patent application 302,758 refers to the use of modified forms of E2 protein that bind to, and block, E2 binding sites on papillomavirus DNA without resulting in trans-activation. That application also refers to repression of E2 activation through the use of DNA fragments that mimic E2 binding sites, and thus bind with E2 trans-activators, making them unavailable for binding to E2 sites on the viral DNA.

U.S. Patent 5,219,990 describes the use of E2 trans-activation repressors which interfere with normal functioning of the native full-length E2 transcriptional activation

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protein of the papillomavirus. However, the E2 trans-activation repressors of the '990 patent are proteins that dimerize with the full-length native E2 protein to form inactive heterodimers, thus interfering with the formation of active homodimers comprising full-length native E2 polypeptides and thereby repressing papillomavirus transcription and replication. The E2 trans-activation repressors are described as fragments of the E2 polypeptide in which the dimerization function has been separated from its DNA binding function, e.g., the E2 trans-activation repressors includes at least the dimerization region, but less than the DNA binding domain, of the E2 polypeptide.

10 *Summary of the Invention*

One aspect of the present invention provides a method of treating, e.g., lessening the severity or preventing the occurrence of, a papillomavirus-induced condition. In general, the subject method comprises administering to an animal, e.g. a human, infected with a papillomavirus a pharmaceutical preparation comprising a therapeutically effective amount of either (i) an E2_{ad/db} polypeptide or (ii) a gene construct for expressing the E2_{ad/db} polypeptide. As described in further detail below, the E2_{ad/db} polypeptide includes a DNA binding domain and a transcriptional activation domain derived from one or more E2 proteins. The E2 polypeptide or gene construct is formulated in the pharmaceutical preparation for delivery into PV-infected cells of the animal.

In preferred embodiments, the subject method is used to treat a human who is infected with a human papillomavirus (HPV), particularly a high risk HPV such as HPV-16, HPV-18, HPV-31 and HPV-33. However, treatment of low risk HPV conditions is also specifically contemplated.

In certain preferred embodiments, the DNA binding and transcriptional activation domains of the E2_{ad/db} polypeptide have amino acid sequences corresponding to an E2 protein(s) from an HPV, especially an E2 protein from a high risk HPV. The DNA binding domain and transcriptional activation domain of the E2 polypeptide can be one contiguous polypeptide chain, or in those embodiments where the E2 protein is directly formulated into the therapeutic composition, the DNA binding and transcriptional activation domain portions of the therapeutic E2 polypeptide can be provided as two separate peptide chains which have been chemically cross-linked, e.g., other than by a amide bond. The E2_{ad/db} polypeptide can be a full length E2 protein, e.g., also including a hinge region sequence or the like, or can lack other E2 peptide sequences except for the DNA binding and transcriptional activation domains.

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The subject method can be used to inhibit pathological progression of papillomavirus infection, such as preventing or reversing the formation of warts, e.g. Plantar warts (*verruca plantaris*), common warts (*verruca plana*), Butcher's common warts, flat warts, genital warts (*condyloma acuminatum*), or epidermodysplasia verruciformis; as well as treating papillomavirus-infected cells which have become, or are at risk of becoming, transformed and/or immortalized, e.g. cancerous, e.g. a laryngeal papilloma, a focal epithelial, a cervical carcinoma.

Another aspect of the present invention relates to a pharmaceutical preparation comprising a therapeutically effective amount of a recombinant transfection system for ameliorating a papillomavirus-induced condition in a subject. For instance, the transfection system, which is for gene therapy, includes a gene construct having a nucleic acid encoding an E2_{ad/db} polypeptide and operably linked to a transcriptional regulatory sequence for causing expression of the E2 polypeptide in eukaryotic cells. The gene construct is provided in a gene delivery composition for delivering the gene construct to a papillomavirus infected cells and causing the cell to be transfected with the gene construct. For example, the gene delivery composition can be, e.g., a recombinant viral particle, a liposome, or a poly-cationic nucleic acid binding agent.

Yet another aspect of the invention relates to a pharmaceutical preparation comprising a therapeutically effective amount of an E2_{ad/db} polypeptide, formulated in the pharmaceutical preparation for delivery into PV-infected cells of an animal. In preferred embodiments, the polypeptide is formulated as a liposome.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds.,

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Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

5 ***Brief Description of the Figures***

Figure 1 schematically illustrates the growth suppression by the full length BPV E2 protein, but not E2-TR. The gross domain structure of the papillomavirus E2 proteins is also illustrated.

10 Figure 2 is a map of an illustrative E2-encoding adenoviral vector suitable for gene therapy.

Figures 3A and 3B illustrate the results of mutational analysis of E2 proteins. Figure 3A shows that the E2 hinge region is not required for growth suppression. Figure 3B shows that the E2 transactivation domain, and a functional E2 DNA binding domain are both required for growth arrest. The properties of the various E2 proteins are also
15 listed

Detailed Description of the Invention

The papillomaviruses (PV) are infectious agents that can cause benign epithelial tumors, or warts, in their natural hosts. Infection with specific human papillomaviruses (HPV) is also associated with human epithelial malignancies, including malignancies of
20 the uterine cervix, genitalia, skin and, though less frequently, other sites.

The analysis of the temporal expression of mRNAs and the nucleotide sequence of human and animal papillomavirus (PV) genomes has revealed overall structural similarities in their genetic organization. There are at least seven open reading frames (ORFs) in all PVs, and differential RNA splicing provides the capability to produce
25 several additional proteins. The genes that are required for viral replication are typically designated with the prefix "E" (for "early"), being expressed before the "L" or "late" genes. The products of the papillomavirus E2 open reading frame play a key role in the regulation of the viral cycle, affecting both transcription and replication. For instance,
30 the E2 transcriptional activation protein (herein the "E2 protein") is a trans-acting factor that activates transcription through specific binding to cis-acting E2 enhancer sequences. The 410 amino acid papillomavirus E2 protein has been shown to induce promoter expression in a classical enhancer mechanism (Spalholz et al. *Cell* (1985) 42:183-91). The E2 protein appears to provide both positive and negative feedback loops for viral
35 gene expression, and appears to be part of a necessary mechanism for achieving long-term persistence of infection.

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As with other transcription factors, the functions of the E2 protein appear to be localized to discrete modular domains (Giri et al. (1988) *EMBO J.* 7:2823-2829). The C-terminal domain of the E2 polypeptide is responsible for recognition of E2 binding sites on viral DNA, and is accordingly referred to herein as the "DNA binding domain".

- 5 The N-terminal domain of the E2 polypeptide is responsible for transcriptional activation following binding of the protein to viral DNA, and is referred to as the "transactivation domain".

In bovine papillomavirus models, and in some human papillomaviruses, at least two N-terminally truncated E2 proteins occur naturally and act as native repressors. It has been experimentally confirmed *in vitro* that truncated forms of E2 proteins which retain their ability to bind DNA but do not trans-activate, are competitive inhibitors of trans-activation-competent E2 polypeptides (Lambert et al. (1987) *Cell* 50:69-78; Stenlund et al. (1990) *Genes Dev.* 4:123-136; and Choe et al. (1989) *J. Virol.* 63:1743-1755).

- 15 As described in the appended examples, we have discovered that HPV E2 proteins which retain both transactivation and DNA binding domains ("E2_{ad/db}" proteins) are capable of inhibiting cell growth of HPV-infected and/or HPV-transformed cells. On the other hand, contrary to certain teachings in the art, we observed that growth of both HPV-infected and HPV-transformed cells was not apparently inhibited by the E2 transcriptional repressor (E2-TR) which represents the DNA binding domain of the E2 protein. This result was somewhat surprising because previous studies found that N-terminally deleted forms of both the HPV and BPV forms of the E2 protein were able to transcriptionally repress the E6/E7 promoter (Thierry et al., (1991) *New Biol.*, 3:90-100). The data presented below suggests that the mechanism for E2-mediated growth suppression may involve more complex mechanisms or that the E2 DNA binding domain is not, in and of itself, an effective repressor of the HPV promoters integrated into the host chromosome.

- Progression of high-risk HPV lesions to cervical cancer is often associated with integration of the viral genome with disruption of the E1/E2 region (Baker C.C. (1993) In: S. O'Brien (ed.) Genetic maps: locus maps of complex genomes, Cold Spring Harbor Laboratory Press, p. 1.134-1.146) his integration leads to the deregulation of the expression of the viral E6 and E7 transforming genes. This may be due in part to the release of the E6/E7 promoter from the repressor effects of E2. The data set forth below demonstrates that the E2 protein can inhibit growth of HPV-immortalized cells.
- 35 Moreover, we have shown that HPV-positive cervical carcinoma cells are sensitive to the reintroduction of HPV E2 proteins; their growth being inhibited by expression of

E2_{ad/db} proteins. While not being bound by any particular theory, the growth-suppressive effect of E2 is presumably mediated by transcriptional repression of E6 and E7 expression from the HPV-16 P₉₇ promoter. However, since mutations in the long control region that eliminate E2 binding in the HPV-16 long control region only partially relieve E2 repression of immortalization, it appears that E2 may affect viral transcription and cellular growth regulation through additional mechanisms.

By virtue of the present invention, there is provided methods and compositions for interfering with the proliferation of cells infected and/or transformed by papillomaviruses. The processes and compositions of this invention may be used to treat any mammal, including humans. According to this invention, mammals are treated by the pharmaceutically acceptable administration of an E2_{ad/db} protein, either directly or by gene transfer techniques, to reduce the symptoms of the specific papillomavirus-associated disease, or to prevent their recurrence. Diseases which may be treated by the processes and compositions of this invention are those caused by the etiological agent, papillomavirus. Such diseases include, for example, epithelial malignancies, anogenital malignancies, such as cervical cancer, malignant lesions, benign lesions, papillomacarcinomas, papilloadenocystomas, papilloma neurophathicum, papillomatosis, cutaneous and mucosal papillomas, condylomas, oral, pharyngeal, laryngeal, and tongue papillomas, fibroblastic tumors and other pathological conditions associated with papillomavirus. The E2-derived compositions of this invention may also be used to treat epithelial and internal fibropapillomas in animals.

A wide variety of warts are found on human skin and are caused by the human papilloma virus (HPV). For example, the following types of warts are found on human skin and are caused by the human papilloma virus (HPV): common warts (*verruca vulgaris*), plantar warts, palmar warts, planar warts (*verruca plana*), mosaic warts, and venereal warts (*condyloma accuminatum*). These skin growths are unsightly, irritating, and potentially oncogenic (carcinogenic), and their removal is desired.

Genital warts, also referred to as venereal warts and *condylomata acuminata*, are one of the most serious manifestations of HPV infection. As reported by the Center for Disease Control, the sexual mode of transmission of genital warts is well established and the incidence of genital warts is on the increase. The seriousness of genital warts is underlined by the finding that HPV DNA can be found in all grades of cervical intraepithelial neoplasia (CIN I-III) and that a specific subset of HPV types can be found in carcinoma *in situ* of the cervix. Consequently, women with genital warts, containing specific HPV types are now considered at high risk for the development of cervical cancer. Current treatments for genital warts are inadequate. According to the present

invention, a method of treating a patient having one or more genital warts comprises the administration of a pharmaceutical composition including an E2_{ad/db} polypeptide, or a gene construct encoding the E2 protein, so as to inhibit growth of the wart. In preferred embodiments, the wart(s) are contacted directly with the pharmaceutical composition.

- 5 The subject method can be used to treat, e.g., condyloma acuminata and/or flat cervical warts.

Laryngeal papillomas are benign epithelial tumors of the larynx. Two PV types, HPV-6 and HPV-11, are most commonly associated with laryngeal papillomas.

- 10 According to the method of the present invention, laryngeal papillomas are treated administering a pharmaceutical composition including the therapeutic E2 polypeptide, or a gene construct encoding the E2 polypeptide, so as to inhibit growth of the papillomas.

- 15 The most common disease associated with papillomavirus infection are benign skin warts. Common warts generally contain HPV types 1, 2, 3, 4 or 10. These warts typically occur on the soles of feet, plantar warts, or on the hands. Common skin warts are most often found in children and young adults. Later in life the incidence of common warts decreases presumably due to immunologic and physiologic changes. Plantar warts can often be debilitating and require surgical removal and they frequently reoccur after surgery. As above, patients suffering from common warts can be treated
- 20 by the administration of a effective amount of an E2 protein according to the present invention, or a gene therapy construct which encodes the therapeutic E2 protein. In preferred embodiments, the protein or gene construct are applied, in the appropriate formulations, directly to the area of the skin afflicted with the wart(s). Similar methods and compositions may be useful in the treatment of epidermodysplasia verruciformis
- 25 (EV), a rare genetically transmitted disease which is characterized by disseminated flat warts that appear as small reddish macules.

In addition, the subject method and compositions may be used to treat lesions resulting from cellular transformation for which HPV is a etiological agent, e.g., in the treatment of cervical cancer.

- 30 For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

- As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or
- 35 DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

As used herein, the term "E2 gene" or "recombinant E2 gene" refers to a nucleic acid comprising an open reading frame encoding a papillomavirus E2 polypeptide.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.

5 "Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of one of an E2 gene is under the control of a promoter sequence (or other transcriptional regulatory
10 sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring forms of E2 proteins.

15 The term "gene construct", with respect to the subject E2 proteins, refers to a vector, plasmid, viral genome or the like which includes an E2 coding sequence, can transfect cells, preferably mammalian cells, and can cause expression of the E2 coding sequence in cells transfected with the construct. The term "gene construct" does not include a wild-type papillomavirus genome, and preferably does not include
20 expressible coding sequences for one or more of a papillomavirus E6 or E7 proteins.

As used herein, the term "tissue-specific promoter" means a DNA sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of hepatic or pancreatic origin, e.g. neuronal cells.
25 The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well.

As used herein, the term "pharmaceutically acceptable" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not toxic to the hosts to which it is administered. The
30 administration(s) may take place by any suitable technique, including subcutaneous and parenteral administration, preferably parenteral. Examples of parenteral administration include intravenous, intraarterial, intramuscular, and intraperitoneal, with intravenous being preferred.

As used herein, the term "prophylactic or therapeutic" treatment refers to
35 administration to the host of the papillomavirus medicament. If it is administered prior to exposure to the virus, the treatment is prophylactic (i.e., it protects the host against

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infection), whereas if administered after infection or initiation of the disease, the treatment is therapeutic (i.e., it combats the existing infection or cancer).

As used herein the term " papillomavirus disease" refers to any kind of disease caused by the virus, including cancers and warts.

5 The term "cell-proliferative disorder" denotes malignant as well as nonmalignant cell populations which morphologically often appear to differ from the surrounding tissue.

Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence.

10 Regulatory sequences are art-recognized and are selected to direct expression of the subject papillomavirus E2 proteins. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a

15 wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding papillomavirus E2 polypeptides of this invention. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the

20 Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters

25 of acid phosphatase, e.g., Pho5, the promoters of the yeast a-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the

30 type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In one embodiment, the expression vector includes a recombinant gene encoding a peptide having an agonistic activity of a subject E2 polypeptide, or alternatively, encoding a peptide which is an

35 antagonistic form of the E2 protein. Such expression vectors can be used to transfect cells and thereby produce polypeptides, including fusion proteins, encoded by nucleic acids as described herein.

1. Therapeutic E2 proteins

The E2_{ad/db} proteins utilized therapeutically by the present invention include both an activation domain (ad) and a DNA binding/dimerization domain (db). As
5 described in the appended examples, the E2 transactivation and DNA binding/dimerization domains were each necessary for E2-mediated growth suppression of PV-infected and PV-transformed cells. E2 mutants which lacked a hinge region, e.g., but which retained the transactivation and DNA binding/dimerization domains, while
10 defective in replication function nevertheless retained the ability to suppress growth of PV-transformed cells, indicating that the growth-suppressive properties of E2 can be unlinked from its DNA replication properties.

The general structure of the papillomavirus E2 protein is well known. See, for example, McBride et al. (1991) *J Biol Chem* 266:18411-18414; and Giri et al. (1988) *EMBO J* 7:2823-2829. Both the activation domain and the DNA binding domain of a
15 papillomavirus E2 protein will be readily identified by one of ordinary skill in the art. For instance, a region of approximately 200 amino acids at the N terminus of E2 corresponds to the activation domain (Figure 1). This domain contains two regions predicted to form acidic amphipathic helices (e.g., using the algorithms developed by Chou and Fasman), which have been shown to be important for the activation function
20 of E2. DNA binding function is provided by the C-terminal 85 amino acids or so of the E2 protein (Figure 1). Dimerization is also mediated by the DNA binding domain. The activation and DNA binding domains are recognized in human papillomavirus E2 proteins, including E2 proteins from the high risk viruses, e.g., HPV-16, -18, -31 or -33.

According to the present invention, the therapeutic E2 protein of the subject
25 method includes both the activation and DNA binding domains. To illustrate, an exemplary E2_{ad/db} protein suitable for use in the present method is generated with a human papillomavirus E2 protein, and preferably from a high risk HPV. For instance, therapeutic compositions of the present invention can be derived from an HPV-16 E2 protein and includes an activation domain corresponding to approximately Met1-Ser198
30 of SEQ ID No. 2 and a DNA binding domain corresponding to approximately Cys281-Ile365 of SEQ ID No. 2. Likewise, the subject method can employ an E2 protein which includes an HPV-18 E2 activation domain corresponding to approximately Met1-203 and a DNA binding domain corresponding to approximately Cys282-Met365 of SEQ ID No. 4.

35 The examples set forth below illustrate that the hinge region (Figure 1), which interrupts the E2 polypeptide sequence between the activation and DNA binding

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domains, is not necessary for the growth suppressive activity of the papillomavirus E2 protein. While in preferred embodiments an intact E2 protein is utilized in the present method, e.g., a contiguous polypeptide corresponding to the activation domain, hinge region and DNA binding domain, the present invention also contemplates the use of E2 proteins which lack all or a portion of the hinge region. For example, a recombinant E2 protein can be provided in which the hinge region is deleted, the activation and DNA binding domains being directly contiguous with one and other. The examples provide the illustrative E2 $_{\Delta 220-309}$ protein to demonstrate the growth inhibitory activity of such constructs. Methods of making fusion proteins (or deletions as the case may be) are well known in the art. Essentially, the joining of various DNA fragments coding for different domains is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the E2 $_{ad/db}$ gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate the E2 $_{ad/db}$ gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

In some instances where the hinge region has been deleted it may be desirable to introduce an unstructured polypeptide linker region between the DNA binding domain and the activation domain in place of the naturally occurring hinge region. This linker can facilitate enhanced flexibility of the protein allowing the two domains to freely and (optionally) simultaneously interact with a DNA and cellular proteins by reducing steric hindrance between the two domains, as well as allowing appropriate folding of each portion to occur. The linker can be of natural origin, such as a sequence determined to exist in random coil between two domains of a protein. Alternatively, the linker can be of synthetic origin. For instance, the sequence (Gly $_4$ Ser) $_3$ can be used as a synthetic unstructured linker. Linkers of this type are described in Huston et al. (1988) PNAS 85:4879; and U.S. Patent Nos. 5,091,513 and 5,258,498, and can be readily incorporated by recombinant techniques.

In still other embodiments, the activation and DNA binding domains can be provided in the same molecule by chemical cross-linking. For example, there are a large number of chemical cross-linking agents that are known to those skilled in the art and useful for cross-linking the activation and DNA binding domains of an E2 protein to

provide a single molecule. In an exemplary embodiment, the cross-linking agents are heterobifunctional cross-linkers which can be used to link the activation and DNA binding domains in a stepwise manner. Heterobifunctional cross-linkers provide the ability to design more specific coupling methods for conjugating proteins, thereby
5 reducing the occurrences of unwanted side reactions such as homo-protein polymers. A wide variety of heterobifunctional cross-linkers are known in the art. These include: succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), m-Maleimidobenzoyl-N-hydroxy-succinimide ester (MBS); N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), succinimidyl 4-(p-maleimido-phenyl)butyrate
10 (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC); 4-succinimidylloxycarbonyl-a-methyl-a-(2-pyridyldithio)-tolune (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP). Those cross-linking agents having N-hydroxysuccinimide moieties can be obtained as the N-hydroxy-sulfosuccinimide analogs, which generally
15 have greater water solubility. In addition, those cross-linking agents having disulfide bridges within the linking chain can be synthesized instead as the alkyl derivatives so as to reduce the amount of linker cleavage *in vivo*.

In addition to the heterobifunctional cross-linkers, there exists a number of other cross-linking agents including homobifunctional and photoreactive cross-linkers.
20 Disuccinimidyl suberate (DSS), bismaleimidohexane (BMH) and dimethylpimelimidate·2 HCl (DMP) are examples of useful homobifunctional cross-linking agents, and bis-[β-(4-azidosalicylamido)ethyl]disulfide (BASED) and N-succinimidyl-6(4'-azido-2'-nitrophenyl)- amino)hexanoate (SANPAH) are examples of useful photoreactive cross-linkers for use in generating the subject E2_{ad/db} proteins. For
25 a recent review of protein coupling techniques, see Means et al. (1990) *Bioconjugate Chemistry* 1:2-12, incorporated by reference herein.

One particularly useful class of heterobifunctional cross-linkers, included in the representative lists above, contain the primary amine reactive group, N-hydroxysuccinimide (NHS), or its water soluble analog N-hydroxysulfosuccinimide
30 (sulfo-NHS). Primary amines (lysine epsilon groups) at alkaline pH's are unprotonated and react by nucleophilic attack on NHS or sulfo-NHS esters. This reaction results in the formation of an amide bond, and release of NHS or sulfo-NHS as a by-product. Another reactive group useful as part of a heterobifunctional cross-linker is a thiol reactive group. Common thiol reactive groups include maleimides, halogens, and
35 pyridyl disulfides. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.5-7.5) conditions. Halogens

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(iodoacetyl functions) react with -SH groups at physiological pH's. Both of these reactive groups result in the formation of stable thioether bonds.

Preparing conjugates of the E2 protein domains using heterobifunctional reagents will typically be a two-step process involving the amine reaction and the
5 sulfhydryl reaction. For the first step, the amine reaction, the E2 polypeptide sequence should contain a primary amine. This can be lysine epsilon amines or an unprotected primary α -amine found at the N-terminus. The polypeptide should not contain free sulfhydryl groups. The polypeptide chain can be modified so that all sulfhydryls are blocked using for instance, N-ethylmaleimide (see Partis et al. (1983) *J. Pro. Chem.*
10 2:263; and Riddles et al. (1979) *Anal. Biochem.* 94:75).

The reaction buffer should be free of extraneous amines and sulfhydryls. The pH of the reaction buffer should be 7.0-7.5. This pH range prevents maleimide groups from reacting with amines, preserving the maleimide group for the second reaction with
15 sulfhydryls. The NHS-ester containing cross-linkers have limited water solubility. They should be dissolved in a minimal amount of organic solvent (DMF or DMSO) before introducing the cross-linker into the reaction mixture. The cross-linker/solvent forms an emulsion which will allow the reaction to occur.

The sulfo-NHS ester analogs are more water soluble, and can be added directly to the reaction buffer. Buffers of high ionic strength should be avoided, as they have a
20 tendency to "salt out" the sulfo-NHS esters. To avoid loss of reactivity due to hydrolysis, the cross-linker is added to the reaction mixture immediately after dissolving the protein solution.

Once the reaction is completed, the first polypeptide portion of the E2 protein is now activated with a sulfhydryl reactive moiety. The activated protein may be isolated
25 from the reaction mixture by simple gel filtration or dialysis. To carry out the second step of the cross-linking, the sulfhydryl reaction, the second E2 polypeptide portion must contain a free sulfhydryl, e.g., an unprotected cysteine residue.

Maleimides react specifically with -SH groups at slightly acidic to neutral pH ranges (6.5-7.5). A neutral pH is sufficient for reactions involving halogens and pyridyl
30 disulfides. Under these conditions, maleimides generally react with -SH groups within a matter of minutes. Longer reaction times are required for halogens and pyridyl disulfides. The first sulfhydryl reactive-protein prepared in the amine reaction step is mixed with the second E2 polypeptide fragment under the appropriate buffer conditions. The E2_{ad}-E2_{db} conjugates can be isolated from the reaction mixture by methods such as
35 gel filtration or by dialysis.

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Generally, where purified stocks of an E2 protein (or fragments thereof) are required, e.g., for formulation intended for direct administration, conventional recombinant techniques can be employed to express and purify an E2 protein. The term "recombinant E2 protein" refers to an E2 polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally DNA encoding an E2_{ad/db} polypeptide (or discrete portions thereof) is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "corresponding to", with respect to a recombinant E2 gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence(s) native E2 polypeptides, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions of a naturally occurring form of an E2 protein.

For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding an E2 polypeptide can be cultured under appropriate conditions to allow expression of the polypeptide to occur. The E2 polypeptide may include a signal sequence and be secreted and isolated from a mixture of cells and medium containing the recombinant E2 polypeptide. Alternatively, the polypeptide may be retained cytoplasmically, as it normally is, and the cells harvested, lysed and the protein isolated. The recombinant E2 polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and affinity purification.

Thus, a nucleotide sequence derived from the cloning of an E2 open reading frame (ORF), encoding all or a selected portion of the full-length protein, can be used to produce a recombinant form of an E2 polypeptide via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well-known PV proteins, e.g. E6, E7 and the like. Similar procedures, or modifications thereof, can be employed to prepare recombinant E2 polypeptides by microbial means or tissue-culture technology in accord with the compositions of the subject invention.

Moreover, it is widely appreciated that fusion proteins can facilitate the expression of proteins, and accordingly, can be used in the expression of the an E2_{ad/db} polypeptides of the present invention. For example, E2_{ad/db} polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins

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can enable easy purification of the E2_{ad/db} polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-

5 (His)/enterokinase cleavage site sequence, can be used at the N-terminus of the E2_{ad/db} protein in order to permit purification of the poly(His)-E2_{ad/db} protein by affinity chromatography using a Ni²⁺ metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

10 In the instance of direct delivery of a therapeutic protein (described *infra*), some uptake mechanisms for the protein may involve passage through lysosomes. As long half-life in the target cells is desirable, an E2 protein of this invention may be modified to increase its resistance to protease degradations and/or acid hydrolysis and, in turn, increase the half-life of the polypeptide in circulation and cells. In one embodiment of
15 the present invention, the protease resistance of an E2 protein is increased by incorporation of D-amino acids instead of L-amino acids at some or all residues of the polypeptide, e.g., as a retro-enantio or retro-inverso peptide. In another embodiment, the peptide backbone is modified using other amide mimetics, such as trans-olefins (Shue et al. (1987) *Tetrahedron Letters* 28:3225) or phosphate derivatives (Loots et al. in
20 *Peptides: Chemistry and Biology*, (Escom Science Publishers, Leiden, 1988, p. 118)) in order to protect the E2 protein from degradation. In still another embodiment, the amino terminus, or carboxy terminus, or both termini of an E2 polypeptide are blocked by chemical modification. In a further embodiment of this invention, lysosomal proteases are inhibited by an E2 protein in a composition comprising a lysomotrophic agent, such
25 as chloroquine, amantadine, monensin, methylamine, or ammonium chloride.

II. Gene Therapy

In one aspect, the present invention relates to gene therapy constructs containing a nucleic acid encoding a papillomavirus E2_{ad/db} polypeptide, preferably an HPV E2
30 protein, operably linked to at least one transcriptional regulatory sequence. The gene constructs of the present invention are formulated to be used as a part of a gene therapy protocol to deliver the subject therapeutic protein to a papillomavirus-infected or -transformed cell in an animal.

Any of the methods known to the art for the insertion of DNA fragments into a
35 vector may be used to construct expression vectors consisting of appropriate transcriptional/ translational control signals and the desired E2-encoding nucleotide

sequence. See, for example, Maniatis T., Fritsch E.F., and Sambrook J. (1989): *Molecular Cloning (A Laboratory Manual)*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; and Ausubel F.M., Brent R., Kingston R.E., Moore, D.D., Seidman J.G., Smith J.A., and Struhl K. (1992): *Current Protocols in Molecular Biology*, John Wiley & Sons, New York. These methods may include *in vitro* DNA recombinant and synthetic techniques and *in vivo* genetic recombination. Expression of a nucleic acid sequence encoding an E2_{ad/db} protein may be regulated by a second nucleic acid sequence so that the protein is expressed in a host infected or transfected with the recombinant DNA molecule. For example, expression of E2 may be controlled by any promoter/enhancer element known in the art. The promoter activation may be tissue specific or inducible by a metabolic product or administered substance.

Promoters/enhancers which may be used to control the expression of the E2 gene *in vivo* include, but are not limited to, the native E2 promoter, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human β -actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

Expression constructs of the subject E2 polypeptides may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the E2 gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO₄ precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will

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depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid encoding the particular E2 polypeptide desired.

- 5 Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., the recombinant E2 protein, are expressed efficiently in cells which have taken up viral vector nucleic acid.

- 10 One viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors for *in vivo* expression of an E2 protein. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *Biotechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155.

- 15 Adenoviruses (Ads) are a relatively well characterized, homogeneous group of viruses. Roughly 100 different adenoviruses, including nearly 50 serotypes isolated from humans, have been identified to date.

- Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled
20 in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they can be used to infect a wide variety of cell types, including epithelial cells (see, for example, Goldman et al. (1995) *J Virol* 69:5951-8; Clayman et al. (1985) *Cancer Gene Therapy* 2:105-111; and Rosenfeld et al. (1992), *supra*), including genitourinary epithelia (Bass et al. (1995) *Cancer Gene Therapy* 2:97-104). Furthermore, the virus
25 particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes
30 integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267).

- Most replication-defective adenoviral vectors currently in use and therefore
35 favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al.

(1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al. in *Methods in Molecular Biology*, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted papillomavirus E2 gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Ad vectors of the present invention can be constructed by ligation of an E2_{ad/db} coding sequence with adenoviral sequences contained in bacterial plasmids. See, for example, Berkner et al. (1983) *Nucleic Acids Res.* 11: 6003-6020; Haj-Ahmad et al. (1986) *J. Virol.* 57: 267-274; and Stow (1981) *J. Virol.* 37: 171-180. In a preferred strategy, two plasmids which together contain sequences comprising the entire Ad genome and the E2_{ad/db} sequence are recombined. A number of conditionally defective plasmid systems have been developed making the construction of vectors simpler and reducing the number of subsequent analyses required to identify recombinant viruses. McGrory et al. (1988) *Virol.* 163: 614-617; Ghosh-Choudhury et al. (1986) *Gene* 50: 161-171; and Mittal et al. (1993) *Virus Res.* 28: 67-90. The Graham et al. PCT publication WO 95/00655, and corresponding Bett et al. (1994) *PNAS* 91:8802-8806 publication, describe a state-of-the-art set of vectors which are useful in generating adenovirus-based vectors and are particularly attractive to generating the E2_{ad/db} expression constructs of the present invention.

The following properties are desirable in the design of an adenovirus vector to transfer the gene for E2 to papillomavirus-transformed cells of a patient. The vector should allow sufficient expression of the E2 protein, while producing minimal viral gene expression. There should be minimal viral DNA replication and ideally no virus replication. Finally, recombination to produce new viral sequences and complementation to allow growth of the defective virus in the patient should be minimized. An exemplary adenovirus vector encoding E2 (Ad5/E2) is described below.

Figure 2 is a map of an illustrative Ad5/E2 construct. This vector includes viral DNA derived from the common relatively benign adenovirus 5 serotype. The E1a and E1b regions of the viral genome, which are involved in early stages of viral replication have been deleted, as has the E3 region (though this is optional). The therapeutic papillomavirus E2 coding sequence is inserted into the viral genome in place of the E1a/E1b region (Δ E1) and transcription of the E2 sequence is driven by the human immediate early promoter region (pCMV) of the human cytomegalovirus.

Merely for illustration, the E2 ORF of a papillomavirus, such as an HPV, are amplified with primers which add HindIII and XbaI restriction sites to the 5' and 3' ends of the ORF, respectively. For example, as described by Del Vecchio et al. (1992) *J*

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Virology 66:5949-5958, PCR can be used to isolate HPV-16 nucleotides 2756 to 3855 (HPV-16 E2) or nucleotides 2817 to 3999 (HPV-18 E2). The amplified E2 coding sequence can then be cloned into the HindIII/XbaI site of the pCDM8 plasmid (Invitrogen catalog V308-20) to provide the E2 coding sequence downstream of pCMV.

- 5 The E2 coding sequence is also flanked at its 3' end by SV40 sequences (SV40intron/pA) which add the transcription termination and polyadenylation signals to an E2 transcript.

The resulting plasmid is linearized with SacII, and the sequence corresponding to the CMV promoter, E2 ORF and SV40intron/pA portion of the plasmid is amplified
10 using primers which add a HindIII site at the 5' end of the CMV promoter and preserve the BamHI site at the 3' end of the SV40 sequences. The resulting PCR product, designated pCMV-E2_{ad/db}-SV40intron/pA, is cleaved by limited digestion with HindIII and BamHI and the appropriate fragment isolated, e.g., which includes the pCMV, E2 ORF and SV40 sequences.

- 15 The resulting HindIII/BamHI fragment is subsequently cloned into the corresponding restriction sites of the pΔE1sp1B vector (see Bett et al. (1994) *PNAS* 91:8802-8806). Following the protocols of Bett et al., the resulting shuttle vector is cotransfected into 293 cells along with, for example, the pHGG10 vector (see Bett et al., *supra*), and infectious particles isolated from the cell culture.

20 Adenoviral vectors currently in use retain most ($\geq 80\%$) of the parental viral genetic material. Recently, second-generation vector systems containing minimal adenoviral regulatory, packaging and replication sequences have therefore been developed and may be used to deliver the therapeutic E2 protein. In one embodiment, the E2 protein is expressed by a pseudo-adenovirus vector (PAV). PAVs contain
25 adenovirus inverted terminal repeats and the minimal adenovirus 5' sequences required for helper virus dependent replication and packaging of the vector. These vectors contain no potentially harmful viral genes, and may be produced in reasonably high titers and maintain the tropism of the parent virus for dividing and non-dividing human target cell types.

30 The PAV vector can be maintained as either a plasmid-borne construct or as an infectious viral particle. As a plasmid construct, PAV is composed of the minimal sequences from the wild type adenovirus necessary for efficient replication and packaging of these sequences and any desired additional exogenous genetic material, by either a wild-type or defective helper virus.

- 35 In one embodiment, an exemplary PAV contains adenovirus 2 (Ad2) sequences including nucleotides (nt) 0-356 of Ad2 forming the 5' end of the vector and the last 109

nt of Ad2 forming the 3' end of the construct. These sequences include the Ad2 flanking inverted terminal repeats and the 5' ITR adjoining sequences containing the known packaging signal and Ela enhancer. PCT publication WO94/12649 describes various PAVs in which convenient restriction sites have been incorporated into the fragments, allowing the insertion of E2-encoding sequences which can be packaged in the PAV virion and used for gene transfer (e.g. for gene therapy). The construction and propagation of PAV is described in detail in WO94/12649. By not containing most native adenoviral DNA, the PAVs are less likely to produce a patient immune response or to replicate in a host.

In addition, the PAV vectors can accommodate foreign DNA up to a maximum length of nearly 36 kb. The PAV vectors therefore, while especially useful for cloning larger genes can be used to transfer more than one gene, or more than one copy of the E2_{ad/db} gene. For example, PAVs can be used to deliver the therapeutic E2 gene in conjunction with other genes such as p21^{CIP1} or p27^{KIP1}.

Yet another viral vector system useful for delivery of a papillomavirus E2 gene is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). One advantageous feature of AAV derives from its reduced efficacy for transducing primary cells relative to transformed cells (Halbert et al. (1995) *J. Virol.* 69:1473). Thus, the spectrum of infectivity for a recombinant E2-derived AAV will be somewhat restricted to papillomavirus transformed cells rather than surrounding normal tissue.

Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

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Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding an E2 protein rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in *Current Protocols in Molecular Biology*, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψ Crip, ψ Cre, ψ 2 and ψ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells (see for example, Shillito et al. (1994) *Cancer Gene Ther* 1:193-204; Noel et al. (1994) *Pediatr Gastroenterol Nutr* 19:43-9; Archer et al. (1995) *PNAS* 91:6840-4; Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and WO94/06920). For instance, strategies for the
5 modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julan et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the
10 chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector in to an amphotropic vector.

15 Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the E2 gene of the retroviral vector.

Numerous retroviral gene delivery vehicles may be utilized within the context of the present invention, including for example EP 0,415,731; WO 90/07936; WO
20 91/0285; WO 9403622; WO 9325698; WO 9325234; U.S. Patent No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart, *Cancer Res.* 53:3860-3864, 1993; Vile and Hart, *Cancer Res.* 53:962-967, 1993; Ram et al., *Cancer Res.* 53:83-88, 1993; Takamiya et al., *J. Neurosci. Res.* 33:493-503, 1992; Baba et al., *J. Neurosurg.* 79:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805). For
25 example, retroviral gene delivery vehicles of the present invention may be readily construction from a wide variety of retroviruses including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (see RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B"
30 retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C retroviruses. Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC" Rockville, Maryland), or isolated from known sources using commonly available techniques.

35 Particularly preferred retroviruses for the preparation or construction of retroviral gene delivery vehicles of the present invention include retroviruses selected from the

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Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe (1976), *J. Virol.* 19:19-25), Abelson (ATCC No. VR-999), Friend, (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, 5 Harvey Sarcoma and Virus, Rauscher (ATCC NO. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Particularly preferred Rous Sarcoma Viruses include Bratislava, Bryan high titer (e.g., ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard, Carr-Zilber, Engelbreth-Holm, Harris, Prague (e.g., ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (e.g. ATCC Nos. VR-724, VR-10 725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS* 15 82:488, 1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, within one embodiment of the invention, retroviral LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an 20 Avian Leukosis Virus.

In some embodiments, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences including the E2-coding sequence, an origin of second strand DNA synthesis and a 3' LTR, but lacking *gag/pol* or *env* coding sequences. Briefly, Long Terminal Repeats 25 ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological activity of a retrovirus, including for example, promoter and enhancer elements which are loaded within U3. LTRs may be readily identified in the provirus due to their precise duplication at either end of the genome.

30 The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, retroviral tRNA binds to a tRNA binding site by Watson-Crick base pairing, and is carried with retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The 35 tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name

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implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly purine tract, is located just upstream of the 3'LTR.

As an illustration, within one embodiment of the invention construction of retroviral vector constructs which lack *gag/pol* or *env* sequences may be accomplished
5 by preparing retroviral vector constructs which lack an extended packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of nucleotides beyond the minimum core sequence which is required for packaging, that allows increased viral titer due to enhanced packaging. As an example, for the Murine Leukemia Virus MoMLV, the minimum core packaging signal is encoded by the
10 sequence (counting from the 5' LTR cap site) from approximately nucleotide 144, up through the Pst I site (Nucleotide 567). See, for example, the Jolly et al. PCT publication WO95/31566. The extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the *gag/pol* gene (nucleotide 621), and beyond nucleotide 1040. Thus, within this embodiment retroviral vector constructs
15 which lack extended packaging signal may be constructed from the MoMLV by deleting or truncating the packaging signal downstream of nucleotide 567.

For example, the pCDM8 plasmid described above, e.g., containing the pCMV promoter, E2 ORF and SV40intron/pA, can be used to generate the PCR product pCMV-E2 ORF-SV40intron/pA, flanked by EcoRI restriction sites. The PCR product is
20 digested with EcoRI, and the appropriate fragment, e.g., which includes the pCMV, E2 ORF and SV40 sequences, is isolated and recloned into the MoMLV-derived pR2 vector (PCT publication WO95/31566) which has been digested at its unique EcoRI site. Modified pR2 vectors with the E2 insert in the correct orientation are isolated. The retroviral vector is electroporated into a packaging cell line, and infectious particles
25 isolated from the cell culture.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a E2 polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In
30 preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the E2 gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In clinical settings, the gene delivery systems for the therapeutic E2 gene can be
35 introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be

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introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470). In preferred embodiments, the gene therapy construct of the present invention is applied topically to HPV infected or transformed cells of the skin or mucosal tissue. A papillomavirus E2 gene construct can, in one embodiment, be delivered in a gene therapy construct by electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

III. Pharmaceutical Preparations of E2 protein

According to another aspect of this invention, E2_{ad/db} proteins may be administered directly to PV infected cells. Direct delivery of E2 proteins may be facilitated by formulation of the protein in any pharmaceutically acceptable dosage form, e.g., for delivery intratumorally, peritumorally, interlesionally, intravenously, intramuscularly, subcutaneously, periolesionally, or (preferably) topical routes, to exert local therapeutic effects.

Topical administration of the therapeutic is advantageous since it allows localized concentration at the site of administration with minimal systemic adsorption. This simplifies the delivery strategy of the agent to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be applied is far less than that required for other administration routes. Effective delivery requires the agent to diffuse into the affected cells. Successful intracellular delivery of agents not naturally taken up by cells has been achieved by exploiting the natural process of intracellular membrane fusion, or by direct access of the cell's natural transport mechanisms which include endocytosis and pinocytosis (Duzgunes (1985)

Subcellular Biochemistry 11:195-286). Such processes are also useful in the direct delivery and uptake of the subject E2_{ad/db} protein by papillomavirus-infected cells.

In one embodiment, the membrane barrier can be overcome by associating the E2 protein in complexes with lipid formulations closely resembling the lipid composition of natural cell membranes. In particular, the subject E2_{ad/db} proteins are encapsulated in liposomes to form pharmaceutical preparations suitable for administration to living cells and, in particular, suitable for topical administration to human skin. The Yarosh U.S. Patent 5,190,762 demonstrates that proteins can be delivered across the outer skin layer and into living cells, without receptor binding, by liposome encapsulation.

These lipids are able to fuse with the cell membranes on contact, and in the process, the associated E2 protein is delivered intracellularly. Lipid complexes can not only facilitate intracellular transfers by fusing with cell membranes but also by overcoming charge repulsions between the cell membrane and the molecule to be inserted. The lipids of the formulations comprise an amphipathic lipid, such as the phospholipids of cell membranes, and form hollow lipid vesicles, or liposomes, in aqueous systems. This property can be used to entrap the E2 protein within the liposomes.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

Liposomes have been described in the art as *in vivo* delivery vehicles. The structure of various types of lipid aggregates varies, depending on composition and method of forming the aggregate. Such aggregates include liposomes, unilamellar vesicles, multilamellar vesicles, micelles and the like, having particle sizes in the nanometer to micrometer range. Methods of making lipid aggregates are by now well-known in the art. For example, the liposomes may be made from natural and synthetic phospholipids, glycolipids, and other lipids and lipid congeners; cholesterol, cholesterol derivatives and other cholesterol congeners; charged species which impart a net charge to the membrane; reactive species which can react after liposome formation to link additional molecules to the liposome membrane; and other lipid soluble compounds which have chemical or biological activity.

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In one embodiment, pH sensitive liposomes are a preferred type of liposome for use with the present invention. One pathway for the entry of liposomes into cellular cytoplasm is by endocytosis into lysosomes of low pH. Accordingly, liposomes which are stable at neutral pH but release their contents at acidic pH can be used to deliver
5 enzymes into the lysosomes of the cytoplasm, whereupon the contents are released.

Liposomes can be made sensitive to the low pH of the lysosomes by the lipid composition. In particular, pH sensitive liposomes can be prepared by using phospholipids which form lipid bilayers when charged but fail to stack in an ordered fashion when neutralized. An example of such a phospholipid is
10 phosphatidylethanolamine, which is negatively charged above pH 9. The net charge of a phospholipid can be maintained at a pH which would otherwise neutralize the head groups by including charged molecules in the lipid bilayer which themselves can become neutralized. Examples of these charged molecules are oleic acid and cholesteryl hemisuccinate, which are negatively charged at neutral pH but become neutralized at pH
15 5. The effect of combining these together in a lipid bilayer is that at pH 9 all molecules are charged; at pH 7 the net negative charge of the oleic acid and cholesteryl hemisuccinate maintains the stability of the phosphatidylethanolamine, and at pH 5 all components are protonated and the lipid membrane is destabilized. Additional neutral molecules, such as phosphatidylcholine, can be added to the liposomes as long as they
20 do not interfere with stabilization of the pH sensitive phospholipid by the charged molecules.

In another embodiment, the E2 polypeptide is formulated with a positively charged synthetic (cationic) lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), in the form of liposomes, or small vesicles,
25 which can fuse with the negatively charged lipids of the cell membranes of mammalian cells, resulting in uptake of the contents of the liposome (see, for example, Felgner et al. (1987) *PNAS* 84:7413-7417; and U.S. Pat. No. 4,897,355 to Eppstein, D. et al.). Another cationic lipid which can be used to generate E2-containing liposomes is the DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethyl-ammonio)propane (DOTAP) in combination
30 with a phospholipid to form delivery vesicles.

Lipofectin™ (Bethesda Research Laboratories, Gaithersburg, Md.) and/or LipofectAMINE™, commercially available reagents, can be used to deliver the E2 protein directly into cells. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma
35 membrane, and can efficiently deliver functional E2 protein into, for example, keratinocytes. Sells et al. (1995) *Biotechniques* 19:72-76 describe a procedure for

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delivery of purified proteins into a variety of cells using such ploycationic lipid preparations.

5 A significant body of information is emerging regarding the use of other cationic lipids for the delivery of macromolecules into cells. Other suitable lipid vesicles for direct delivery of the E2 protein include vesicles containing a quaternary ammonium surfactant (Ballas et al. (1988) *Biochim. Biophys Acta* 939:8-18); lipophilic derivatives of spermine (Behr et al. (1989) *PNAS* 86:6982-6986).

10 The lipid formulations of the subject E2 protein can be used in pharmaceutical formulations to deliver the E2 protein by various routes and to various sites in the animal body to achieve the desired therapeutic effect. Local or systemic delivery of the therapeutic agent can be achieved by administration comprising application or insertion of the formulation into body cavities, inhalation or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, intradermal, peritoneal, subcutaneous and topical administration.

15 Topical formulations are those advantageously applied to the skin or mucosa. Target mucosa can be that of the gastrointestinal tract, comprising the mouth, larynx, esophagus and stomach, or the vaginal, vulvar, penial or anorectal mucosa. Other target tissues can be the accessible epidermal tissues which are infected by HPV. Lipids present in topical formulations can act to facilitate introduction of therapeutic E2 protein into the target tissue, such as the stratum or corneum of the skin, by perturbing the barrier properties of the protective membrane, or by introducing perturbing agents or penetration enhancers such as Azone™ or by promoting the activity of these penetration enhancers.

25 Other pharmaceutical formulations comprising the cationic lipids of the invention are topical preparations containing an anesthetic or cytostatic agent, immunomodulators, bioactive peptides or oligonucleotides, sunscreens or cosmetics. Preparations for topical use are conveniently prepared with hydrophilic and hydrophobic bases in the form of creams, lotions, ointments or gels; alternatively, the preparation may be in the form of a liquid that is sprayed on the skin. The effect of the cationic lipids is to facilitate the penetration of the active antiviral agent through the stratum corneum of the dermis.

30 The composition and form of pharmaceutical preparations comprising the liposome, in combination with the E2 protein, can vary according to the intended route of administration.

35 Also, by suitable modifications of the liposome membranes, the liposomes can be made to bind to specific sub-populations of cells.

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In still another embodiment, the therapeutic E2 protein can be delivered by way of an artificial viral envelope (AVE). Briefly, the art as described a number of viral envelopes which exploit molecular recognition of cell surface receptors by viral surface proteins as a means for selective intracellular delivery of macromolecules, including proteins. According to the method of Schreier, et. al., U.S. Patent 5,252,348, a virtually unlimited number of artificial viral envelopes can be prepared and applied using recombinant or isolated surface determinants. For example, the AVEs be generated as viral mimetics of a number of human viruses including arboviruses; flaviviridae; bunyaviridae; hepatitis viruses; Epstein-Barr viruses; herpes viruses; paramyxoviruses; respiratory syncytial virus; retroviruses including human T-lymphotrophic virus type I and II (HTLV-I/II) and human immunodeficiency virus type 1 and 2 (HIV- 1/2); rhinoviruses; orthopoxviruses; and human papilloma viruses.

In another embodiment, direct delivery of a therapeutic E2 protein may be facilitated by chemical modification of the polypeptide itself. One such modification involves increasing the lipophilicity of the E2 protein in order to increase binding to the cell surface, in turn, stimulating non-specific endocytosis of the protein. Lipophilicity may be increased by adding a lipophilic moiety (e.g., one or more fatty acid molecules) to the E2 protein. A wide variety of fatty acids may be employed. For example, the protein may be palmitoylated. Alternatively, a lipopeptide may be produced by fusion or cross-linking, to permit the E2 protein to resemble the natural lipopeptide from E.coli, tripalmitoyl-S-glycerylcysteil-seryl-serine, at its amino terminus. This lipopeptide has been shown to increase the uptake of fused peptides (P. Hoffmann et al., (1988) *Immunobiol.* 177:158-70). Lipophilicity may also be increased by esterification of the protein at tyrosine residues or other amino acid residues. And uptake of the E2 protein may be increased by addition of a basic polymer such as polyarginine or polylysine (Shen et al. (1978) *PNAS* 75:1872-76).

Direct delivery of E2 proteins according to this invention may also be effected by the use of transport moieties, such as protein carriers known to cross cell membranes. For example, an E2 protein may be fused to a carrier protein, preferably by a genetic fusion which may be expressed in a system such as E.coli or yeast. According to one embodiment of this invention, the amino terminus of the E2 protein may be fused to the carboxy terminus of a transport moiety using standard techniques.

Nucleotide sequences encoding such carrier-E2 protein fusion proteins, operatively linked to regulatory sequences, may be constructed and introduced into appropriate expression systems using conventional recombinant DNA procedures. The resulting fusion protein may then be purified and tested for its capacity to (1) enter intact

eukaryotic cells and (2) inhibit E2-dependent gene expression and viral DNA replication once inside the intact eukaryotic cells.

In choosing a useful carrier protein, those of skill in the art will recognize the desirability of appropriate control experiments designed, inter alia, to test the possibility
5 that the carrier portion of the fusion protein itself interacts with elements of the E2 transcriptional regulation system. If the carrier portion of the fusion protein is found to have undesirable interactions, such as activation of E2-dependent enhancer elements, the portions of the carrier sequence responsible for these interactions should be identified and deleted in a way which permits the sequence to retain its carrier capacity.
10 Alternately, one of several conventional carrier sequences which do not interact with elements of the E2 transcriptional regulation system can be substituted.

Useful carrier proteins include, for example, bacterial hemolysins or "blending agents", such as alamethicin or sulfhydryl activated lysins. Other carrier moieties which may be used include cell entry components of bacterial toxins, such as *Pseudomonas*
15 exotoxin, tetanus toxin, ricin toxin, and diphtheria toxin. Also useful is melittin, from bee venom. Other useful carrier proteins include proteins which are viral receptors, cell receptors or cell ligands for specific receptors that are internalized, i.e., those which cross mammalian cell membranes via specific interaction with cell surface receptors, recognized and taken into the cell by cell surface receptors. Such cell ligands include, for
20 example, epidermal growth factor, fibroblast growth factor, transferrin and platelet-derived growth factor. Alternatively, the ligand may be a non-peptide, such as mannose-6-phosphate, which permits internalization by the mannose-6-phosphate receptor. The transport moiety may also be selected from bacterial immunogens, parasitic immunogens, viral immunogens, immunoglobulins or fragments thereof that bind to
25 target molecules, cytokines, growth factors, colony stimulating factors and hormones. A transport moiety may also be derived from the tat protein of HIV-1.

As an alternative or addition to the above-described chemical modifications and protein carriers, which may be employed alone or in combination, other agents which allow penetration of the keratinized cell layer may be employed to facilitate delivery of
30 the E2 proteins of this invention to papillomavirus-infected cells. In topical applications, for example, the E2 protein may be administered in combination with dimethylsulfoxide, an agent which promotes penetration of cell membranes by substances mixed with it. Useful keratinolytic agents include, for example, salicylic acid, urea, and alpha -hydroxyacids. For such applications, the E2 protein and any other
35 agent may be administered topically, in cream or gel form.

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According to an alternate embodiment of this invention, the therapeutic E2 protein may be administered serially or in combination with other therapeutics used in the treatment of papillomavirus infections or diseases caused by them. Such therapeutics include interferons, such as IFN- γ , IFN- α and IFN- β derived from natural sources or
5 produced by recombinant techniques, other cell mediators formed by leukocytes or produced by recombinant techniques such as for example, interleukin-1, interleukin-2, tumor necrosis factor, macrophage colony stimulating factor, macrophage migration inhibitory factor, macrophage activation factor, lymphotoxin and fibroblast growth factor. Alternatively, the E2 protein may be administered serially or in combination with
10 conventional therapeutic agents or regimens such as, for example, salicylic acid, podophyllotoxin, retinoic acid, surgery, laser therapy and cryotherapy. Such combination therapies may advantageously utilize less than conventional dosages of those agents, or involve less radical regimens, thus avoiding any potential toxicity or risks associated with those therapies.

15 It will also be understood by those skilled in the art that any of the above enumerated delivery methods may be augmented, where topical application is being carried out, by the use of ultrasound or iontophoretic delivery devices which facilitate transdermal delivery of proteins. See, for example, Banga et al. (1993) *Pharm Res* 10:697-702; and Mitragotri et al. (1995) *Science* 269:850-853.

20

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not
25 intended to limit the invention.

Carcinogenic progression of a human papillomavirus (HPV)-infected cell is often associated with integration of the viral genome in a manner which results in the loss of expression of the viral regulatory protein E2. One function of E2 is the regulation of expression of the viral oncogenes, E6 and E7. Introduction of the bovine
30 papillomavirus type 1 (BPV-1) E2 transactivator (E2-TA) in HeLa cells, an HPV type 18 (HPV-18)-positive cervical carcinoma cell line results in growth arrest. In this study, we have found that the HPV-16 and HPV-18 E2 proteins share with BPV-1 E2-TA the ability to suppress HeLa cell growth. This property was not observed for the BPV-1 E2 transcriptional repressor (E2-TR). Analysis of various mutant E2 proteins for growth
35 suppression revealed a requirement for the intact transactivation and DNA binding domains. A HeLa cell line (HeLa-*tsE2*) which expressed a conditional mutant E2 protein

that was functional only at the permissive temperature (32°C) was established, permitting an analysis of the molecular and cellular consequences of E2 expression. Our data indicates that one mechanism by which E2 suppresses cellular growth is through repression of E6 and E7 expression, thereby enabling the cellular targets of E6 and E7 to resume regulation of the cell cycle.

The functions of the E2 proteins have been extensively characterized for BPV-1. The BPV-1 E2 ORF encodes three different proteins: an internally initiated E2 transcriptional repressor (E2-TR), a spliced product between E8 and E2 (E8-E2), and the E2 transactivator (E2-TA) (Hubbert et al. (1988) *Proc. Natl. Acad. Sci. USA*, 85:5864-5868; Lambert et al. (1989) *J. Virol.*, 63:3151-3154). The full-length E2 protein (E2-TA) can function as a transactivator or a repressor, depending on the location of E2 binding sites in a responsive promoter. It has two functional domains which are well conserved among the E2 proteins of different papillomaviruses (Giri and Yaniv (1988) *EMBO J.*, 7:2823-2829; McBride et al. (1991) *J. Biol. chem.*, 266:18411-18414), a 200-amino-acid transactivation domain at the N terminus and a 90- to 100-amino-acid C-terminal domain that is essential for dimerization and DNA binding (Li et al. (1989) *Genes Dev.*, 3:510-526; McBride et al. (1989) *Proc. Natl. Acad. Sci. USA*, 86:510-514, McBride et al. (1988) *EMBO J.*, 7:533; Moskaluk and Bastia (1988) *J. Virol.*, 62:1925-1931). A flexible spacer region called "the hinge" separates the transactivation and DNA binding domains. Both E2-TR and E8-E2 can inhibit the transactivation function of the full-length protein, through mechanisms that may involve competition with E2-TA for DNA binding or by subunit mixing and heterodimer formation with the full-length protein. E2-TA can directly repress transcription of promoters in which the E2 binding sites are located near essential promoter elements such as the TATA box or SP1 binding sites (Dostatni et al. (1991) *Genes Dev.*, 5:1657-1671; Gloss and Bernard (1990) *J. Virol.*, 64:5577-5584; Matsushima et al. (1994) *Mol. Cell Biol.*, 14:2066-2076; Li et al. (1991) *Cell* 65:493-505, Romanczuk et al. (1990) *J. Virol.*, 64:2849-2859; Spalholz et al. (1991) *J. Virol.*, 65:743-753; Thierry and Howley (1991) *New Biol.*, 3:90-100; Vande Pol and Howley (1990) *J. Virol.*, 64:5420-5429). In these cases, E2-TA is believed to interfere with the assembly of the transcriptional initiation complex.

The studies presented here were designed to examine the effect of E2 expression in cervical carcinoma cell lines. We were able to confirm earlier studies showing that expression of BPV-1 E2-TA results in growth suppression of HeLa cells. However, we found that (i) the expression of HPV E2 proteins specifically inhibited PV-infected and PV-transformed cells, as opposed to the non-specific inhibition by BPV E2 proteins shown in the art, and (ii) the E2-TR protein was unable to suppress cellular growth.

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Using a series of mutated E2 proteins, were able to determine which domains of the E2 protein were important for this activity. We were also able to demonstrate that HPV-16 and HPV-18 E2 proteins had similar growth suppression functions. Establishment of a HeLa cell line allowed further characterization of E2-mediated growth suppression.

- 5 These studies indicate that at least one consequence of E2 expression in HPV-positive cell lines is decreased expression of the viral oncoproteins, resulting in cell cycle arrest.

Materials And Methods

- Recombinant plasmids:* The plasmids used for expression of the various BPV-1
10 E2 proteins were based on C59, which utilizes the SV40 early promoter to express the full-length E2 protein (Spalholz et al. (1991) *J. Virol.*, 65:743-753; Yang et al. (1985) *Nature* (London) 318:575-577). The C59-derived E2-TA plasmid used in this study has been modified to contain a Kozak initiation consensus sequence (CCACCATG [Kozak, M. (1991) *J. Biol. Chem.*, 266:19867-19870]) as previously described (Winokur and
15 McBride (1992) *EMBO J.*, 11:4111-4118). The plasmids used for expression of E2-TR and the E2-TR with the translation termination linker in E5 (p1175) were previously described (Lambert et al. (1987) *Cell* 50:68-78). Plasmids which express the E2 mutant proteins E2_{>1-15}, E2_{>157-282}, E2_{>220-309} (McBride et al. (1989) *Proc. Natl. Acad. Sci. USA*, 86:510-514; Winokur and McBride (1992) *EMBO J.*, 11:4111-4118), and E2₁₋₂₁₈
20 have been previously described (Yang et al. (1985) *Nature* (London) 318:575-577).

- ts E2* was subcloned from plasmid pE2ts-1 (DeMaio and Settleman (1988) *EMBO J.*, 7:1197-1204) by PCR using oligonucleotides containing a HindIII site at the 5' end and an XbaI site at the 3' end of the E2 gene. Pfu polymerase (from Stratagene) was used to ensure high fidelity. The DNA fragment containing temperature-sensitive
25 E2 (*tsE2*) was cloned into the vector pRC-CMV (Invitrogen) at the HindIII-XbaI sites of the polylinker. This vector also contains the gene for neomycin resistance. The sequence of this clone was verified by automated DNA sequence analysis (ABI model 373A sequencer). The mutant E2 gene contains an insertion of the four-amino-acid sequence Pro-Arg-Ser-Arg between amino acids 181 and 182 (DeMaio and Settleman
30 (1988) *EMBO J.*, 7:1197-1204).

- Cell culture:* HeLa, HT-3, SiHa, Caski, and C33A are human cervical cancer cell lines obtained from the American Type Culture Collection that have been previously analyzed for the presence of HPV DNA and HPV RNA (Yee et al. (1985) *Am. J. Pathol.*, 119:361-366). Saos-2, a human osteosarcoma cell line, was obtained from Phil
35 Hinds (Harvard Medical School). An immortalized human foreskin keratinocyte cell line (W16) was immortalized by using the full-length genome of HPV-16 linearized

with BamHI, as previously described (Romanczuk and Howley (1992) *Proc. Natl. Acad. Sci. USA*, 89:3159-3163).

HeLa, SiHa, Caski, C33A, and Saos-2 cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum. HT-3 cells were maintained in
5 McCoy's 5A medium (Gibco/BRL) with 10% fetal bovine serum. The HPV-16-immortalized Keratinocyte line (W16) was maintained in 3+1 medium (3 parts KGM plus 1 part Dulbecco modified Eagle medium).

Growth suppression assay: Cells were seeded at 1×10^6 to 2×10^6 cells per 10-cm-diameter dish the day before transfection. Cells were transfected by lipofection
10 (Lipofectin; GIBCO BRL), using 8 to 10 μ g of plasmid DNA purified through two CsCl gradient centrifugations per 10-cm-diameter plate. Sixteen hours after transfection, cells were referred; at 24h posttransfection, the cells were split and placed under selection in medium containing 10% fetal bovine serum and G418 (concentration dependent on the cell line). Cells were maintained under selection for 2 to 3 weeks until the number of
15 drug-resistant colonies could be determined. Cells were fixed in 10% formaldehyde for 15 min. washed, and stained with methylene blue for 15 min. Plates were washed, and G418-resistant colonies were counted.

Chloramphenicol acetyltransferase assays: Reporter plasmid was cotransfected into HeLa cells (Chen and Okayama (1987) *Mol. Cell Biol.*, 7:2745-2752) with the
20 indicated expression plasmids (empty vector was used to normalize the amount of total DNA transfected) by the calcium phosphate method. At 48h after transfection, cells were harvested and lysed in 250 mM Tris (pH 8.0), and chloramphenicol acetyltransferase activity was determined (Gorman et al. (1982) *Mol. Cell Biol.*, 2:1044-1051).

25 *Generation of HeLa-ts E2 cell line:* ts E2 was linearized with BglII and transfected into HeLa cells by lipofection. Transfected cells containing ts E2 were selected for in 450 μ g of G418 per ml. and individual colonies were analyzed.

RNA analysis: The HeLa-tsE2 cell line and the control cell line containing the vector alone (HeLa-V) were split, and 10^6 cells were plated. Cultures were at
30 maintained at 32 or 38°C for 5 days. Total RNA was harvested by using a Biotex RNA isolation kit. Total RNA (14 μ g) was separated by 1.2% agarose-formaldehyde gel electrophoresis for Northern (RNA) blot analysis. The RNA was transferred to a GeneScreen Plus filter and hybridized with a probe for HPV-18 E6/E7 mRNA levels and with the cyclophilin probe (Danielson et al. (1988) *DNA*, 7:261-267).

35 *Immunological procedures:* For immunoblotting, cell lysates were prepared in lysis buffer (0.1 M NaCl, 2mM EDTA, 20 mM Tris [pH 8.0], 1% Nonidet P-40, per ml,

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5 mM sodium fluoride, 1 mM sodium orthovanadate) on ice for on ice for 30 min. Lysates were cleared by centrifugation at 15,000 x g at 4°C for 5 min. A 100 µg aliquot of protein from the cell lysate of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting (Towbin et al. (1979) *Proc. Natl. Acad. Sci. USA*, 86:4350-4354). The protein concentrations were determined by the Bio-Rad protein assay. Monoclonal antibody 1801 (Ab-2 from Once gene Science) was used for p53 detection. Monoclonal antibody 245 (PharMingen) was used for pRb detection. Mouse monoclonal antibody sdi-1p21 (1509-A; PharMingen) was used for p21/WAF1/Cip1 detection. Horseradish peroxidase-conjugated rabbit anti-mouse antibody (NA 931; Amersham) was used, followed by enhanced chemiluminescence detection (Renaissance system; NEN).

Immunocomplex kinase assays: Immunoprecipitations were carried out essentially as described previously (Matsushime et al. (1994) *Mol. Cell Biol.*, 14:2066-2076), using 100 µg of cellular extract. Rabbit polyclonal antibody C-8 (Santa Cruz) was used to precipitate cyclin A. Mouse monoclonal antibody HE-111 (Santa Cruz) was used to precipitate cyclin E complexes.

Kinase assays were carried out as described previously (Matsushime et al. (1994) *Mol. Cell Biol.*, 14:2066-2076), using histone H1 as a substrate. Proteins were separated by SDS-PAGE (11% polyacrylamide gel). Coomassie blue stained, and dried, and phosphorylated proteins were visualized by autoradiography.

Results

(i) *Suppression of HeLa cell growth by BPV-1 E2-TA but not E2-TR.*

Expression of the full-length BPV-1 E2-TA protein in HeLa cells results in suppression of cell growth as assayed by a reduction in colony formation (Thierry and Yaniv (1987) *EMBO J.*, 6:3391-3397). We believe that the decreased expression of the viral oncoproteins may contribute to this growth suppression, since expression of the full-length E2 proteins of BPV-1, HPV-16, and HPV-18 as well as HPV-18C (an artificially truncated HPV-18 E2 repressor construct, analogous to BPV-1 E2-TR) is capable of down regulating transcription from the HPV-18 P₁₀₅ promoter (Thierry and Howley (1991) *New Biol.*, 3:90-100). Furthermore, growth suppression of HeLa cells by BPV-1 E2-TA expressed from an SV40-based vector results in the down regulation of E6 and E7 expression from the viral P₁₀₅ promoter (Hwang et al. (1993) *J. Virol.*, 67:3720-3729).

To further investigate the mechanisms involved in the E2 regulation of cellular proliferation, we examined whether BPV-1 E2-TR could suppress HeLa cell growth like

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the full-length BPV-1 E2-TA. HeLa cells were cotransfected with a plasmid conferring neomycin resistance (pSV2neo) and a plasmid encoding either BPV-1 E2-TA or BPV-1 E2-TR. Cells were split after transfection and maintained in G418-containing medium for 14 to 21 days, at which time the number of G418-resistant colonies was determined.

- 5 No growth suppression was observed with BPV-1 E2-TR (Figure 1). Since the E2-TR plasmid used in this experiment contains an intact E5 gene which might mask or otherwise affect a growth-suppressive effect of E2-TR, an E2-TR construct (p1175) with a translation termination linker in E5 was tested and also found to be defective for HeLa cell growth suppression. Similar results were obtained when puromycin selection rather
10 G418 selection was used, indicating that these results were not specific for neomycin resistance selection.

The lack of growth suppression by E2-TR was unexpected, since E2-TR has been shown to specifically repress E2-responsive promoters. To confirm that E2-TR was indeed expressed in HeLa cells, the E2-TR plasmid was tested for its ability to
15 repress E2-TA transactivation in HeLa cells. HeLa cells were transfected with the E2-TA plasmid, the E2-responsive plasmid 6XE2TKCAT (Thierry et al. (1990) *Mol. Cell Biol.*, 10:4431-4437), and increasing amounts of the E2-TR plasmid. We observed that E2-TR was able to specifically repress E2-TA transactivation, indicating expression of functional E2-TR in HeLa cells.

20

(ii) *HPV-16 and HPV-18 E2 proteins suppress cell growth in HeLa cervical carcinoma cells.*

The abilities of HPV-16 and HPV-18 E2 proteins to suppress HeLa cell growth were next examined. In this series of experiments, we tested whether the E2 proteins of the
25 high-risk HPVs shared the growth-suppressive function shown for the BPV-1 E2-TA protein. The experiment was performed with HPV-16 E2 and HPV-18 E2 expression plasmids that had been previously shown to express functional E2 protein capable of supporting origin-dependent DNA replication and of activating E2-responsive promoters (Del Vecchio et al. (1992) *J. Virol.*, 66:5949-5958). Both HPV-16 E2 and HPV-18 E2
30 proteins suppressed HeLa cell growth in the colony formation assay (Table 1). This result differs from a previous report that expression of HPV-18 E2 was not able to suppress the growth of HeLa cells (Thierry and Yaniv (1987) *EMBO J.*, 6:3391-3397).

TABLE 1. HeLa cell growth suppression by HPV-16 and HPV-18 E2^a

Transfection	No. of G418-resistant colonies ^b	
	Expt. 1	Expt. 2
HPV-16 E2	0	0
HPV-18 E2	0	0
Vector alone	34	41

5 ^a HeLa cells were transfected with 1 µg of pSV2neo and 10 µg of either HPV-16 E2- or HPV-18 E2-expressing plasmid or vector alone.

^b Transfected cells were maintained under selection in medium containing 500 µg of G418 per ml for 2 to 3 weeks, and the number of G418-resistant colonies from each transfection was determined.

10

(iii) *Growth suppression mediated by E2 is observed in other HPV-positive cell lines.*

To examine the specificity of E2 growth suppression, additional cell lines were assayed for their responses to E2 expression using the colony reduction assay. E2 growth suppression was observed in each of the three HPV-positive cell lines tested in which the viral genes are expressed from the viral long control region. These include two HPV-16-positive cervical carcinoma cell lines. SiHa and Caski, and W16, which is a human foreskin keratinocyte cell line immortalized by HPV-16 (Table 2). Growth suppression by BPV-1 E2-TA was observed in each of these cell lines, and no growth suppressive effect was observed with the BPV-1 E2-TR protein. These data are in agreement with the results of Hwang et al. (Hwang et al. (1993) *J. Virol.*, 67:3720-3729), who also reported growth suppression of other HPV-positive cervical cancer cell lines by BPV-1 E2-TA.

TABLE 2. BPV-1 E2 growth suppression of HPV-16-expressing human cell lines^a

25

Transfection	No. of G418-resistant colonies					
	SiHa		Caski		W16	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
E2-TA	6	4	2	0	4	8
E2-TR	52	42	59	67	18	19
Vector alone	46	57	51	55	24	23

^a Indicated cell lines were transfected with 1 µg of pSV2neo and 10 µg of either BPV-1 E2-TA- or BPV E2-TR-expressing plasmid or vector alone.

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To determine whether E2 can act as a general growth suppressor, two cervical carcinoma cell lines which are HPV negative (C33A and HT-3) and a human osteosarcoma cell line (Saos-2) were tested for growth suppression by E2-TA. It was previously reported that E2-TA could suppress the growth of HT-3 cells when introduced by infection with an E2-expressing recombinant SV40 (Hwang et al. (1993) *J. Virol.*, 67:3720-3729). In our assay, however, the growth of each of these HPV-negative cell lines, including HT-3, was unaffected by expression of the E2-TA protein (Table 3). Thus, the E2 growth suppression in the colony reduction assay is specific for HPV-positive cells.

TABLE 3. Lack of BPV-1 E2 growth suppression of HPV-negative human cell lines^a

Transfection	No. of G418-resistant colonies		
	Saos-2		HT-3
	Expt. 1	Expt. 2	
E2-TA	67	80	24
E2-TR	81	102	28
Vector alone	62	99	19

^a See the footnote to Table 2. For C33A cells, values were >500 in all assays in two experiments.

(iv) *Intact DNA binding and transactivation functions of the E2 protein are required for inhibition of HeLa cell growth.*

The domains of BPV-1 E2 necessary for growth suppression were mapped by using a series of truncation and deletion mutants of E2, which had been previously characterized for their transactivation and replication functions (Winokur and McBride (1992) *EMBO J.*, 11:4111-4118). The growth-suppressive phenotype conferred by the E2 deletion mutants was assayed in HeLa cells by using the colony reduction assay (Figure 3A). Growth suppression was observed with E2-TA and with E2_{>220-309}, which is a mutant deleted of a large segment of the hinge region but containing an intact transactivation domain and an intact DNA binding/dimerization domain. No growth suppression was observed for either E2_{>1-15} or E2_{>157-220}, both of which are deficient for the transcriptional transactivation and DNA replication functions in mammalian cells. Each of these E2 mutants can inhibit the transactivation properties of E2-TA in trans. An E2 mutant protein (E2₁₋₂₁₈) consisting of only the transactivation domain similarly had no negative effect on cell growth. The E2_{>220-309} mutant, which has growth-suppressive activity, is able to transactivate an E2-responsive plasmid but is

defective in origin-dependent DNA replication (Winokur and McBride (1992) *EMBO J.*, 11:4111-4118). We confirmed the transactivation or transrepression properties of each of these mutant E2 proteins in HeLa cells. The expression of the E2 proteins was demonstrated in COS cells by immunoblotting, using expression from recombinant
5 PAVA viruses. In these studies, it was noted that the E2₁₋₂₁₈ mutant was expressed at a somewhat lower level than the other E2 proteins. These results indicate that the BPV-1 E2 growth-suppressive effect in HeLa cells requires a functional transactivation domain as well as an intact DNA binding domain but that an intact DNA replication function is apparently not necessary.

10 The genetic organization of BPV-1 is complex, with overlapping ORFs at the 3' end of the early region, raising the possibility that another ORF contributes to the growth suppression phenotype. This, However, is quite unlikely. The intact E2-TA expression vector that was used contains additional ORFs, including E3 (3267 to 3551), E4 (3173 to 3526), and E5 (3714 to 4010). The E2_{>220-309} deletion mutant plasmid,
15 which expresses an E2 capable of growth suppression, is deleted of most of the coding regions of E3 and E4 (from 3265 to 3532 of the BPV-1 genome). Furthermore, E5 has been previously shown not to be associated with the growth-suppressive effect observed with the BPV-1 E2 expression vector (Thierry and Yaniv (1987) *EMBO J.*, 6:3391-3397). Thus, we conclude that the growth-suppressive phenotype can be attributed to
20 E2.

We next examined the specificity of the E2 transactivation domain in suppressing HeLa cell proliferation by testing whether this function could be provided by other acidic activation domains. Chimeric constructs containing the E2 DNA binding/dimerization domain fused to either the acidic transactivation domain of the
25 herpesvirus VP16 transcriptional activator or the spi oncogene (Gauthier et al. (1993) *EMBO J.*, 12:5089-5096) were tested in the colony reduction assay (Figure 3B). Although both chimeric proteins were able to efficiently transactivate E2-responsive reporter constructs in HeLa cells, neither had the ability to suppress HeLa cell proliferation. This finding indicates that the ability of the E2 transactivation domain to
30 suppress cellular proliferation is specific and not due to its general transactivation property.

The dimerization and DNA binding properties of BPV-1 E2 have been separated by point mutations in the DNA binding domain (Barsoum et al. (1992) *J. Virol.*, 66:3941-3945; McBride et al. (1989) *J. Virol.*, 63:5076-5085). E2 mutants that can
35 dimerize but not bind DNA and that can no longer dimerize or bind DNA have been described. Two of these E2 mutants were tested for the ability to suppress HeLa cell

growth. Neither the E2_(I331R) mutant, which is defective for both dimerization and DNA binding, nor the E2_(R344K) mutant, which can dimerize but not bind DNA, was capable of suppressing HeLa cell proliferation (Figure 3B). These results suggest that both the E2 transactivation and DNA binding functions are necessary for the E2 growth suppression phenotype.

(v) *Molecular consequences of E2 expression.*

To study the downstream effects of E2 expression in HPV-positive cells, we used a conditional BPV-1 E2 mutant that has normal replication and transactivation functions at 32°C which are inactivated at either 37 or 39°C (DiMaio and Settleman (1988) *EMBO J.*, 7:1197-1204). This mutant E2 gene was subcloned behind the cytomegalovirus promoter into an expression vector containing the neomycin resistance gene to generate plasmid pRC-tsE2. This plasmid was introduced into HeLa, SiHa, and C33A cells, and the number of G418-resistant colonies was determined after 21 days at 32 and 38°C (Table 4). Following transfection at the nonpermissive temperature (38°C), the cells were split and incubated at either 32 or 38°C under G418 selection. At the permissive temperature (32°C), tsE2 inhibited colony formation, similar to the effect of the wild-type E2-TA, in both the HeLa and SiHa cell lines. However, at the nonpermissive temperature (38°C), there was no growth inhibition in any of the cell lines tested. In agreement with the results obtained with wild-type E2, no growth suppression was observed with tsE2 in C33A cells at the permissive temperature. Control transfections performed with the pRC-neo vector alone confirmed that the growth suppression observed in HeLa and SiHa cells was not due to temperature, since G418-resistant colonies appeared for both cell lines at 32°C.

TABLE 4. Temperature-sensitive growth phenotype of cells containing the E2 mutant^a

Cell line	No. of G418-resistant colonies			
	pRC-tsE2		pRC-neo	
	32°C	38°C	32°C	38°C
HeLa	0	73	41	61
SiHa	0	86	37	77
C33A	>500	>500	>500	>500

^a Indicated cell lines were transfected with 1 µg of pSV2neo and 10 µg of either BPV-1 tsE2 or vector alone.

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pRC-*tsE2* was used to generate a G418-resistant HeLa cell line at 38°C which expressed the conditional E2 mutant protein (HeLa-*tsE2*). HeLa-*tsE2* clonal lines were characterized for the ability to transactivate an E2-responsive plasmid and for their growth properties at 32 and 38°C. As a control, a G418-resistant HeLa cell line (HeLa-V) was also established by using the vector without insert. The HeLa-*tsE2* cells were growth arrested at 32°C, whereas the HeLa-V cells were not. Fluorescence-activated cell sorting analysis indicated that the HeLa-*tsE2* cells were arrested in G₁ when grown at 32°C, consistent with previously reported data indicating that BPV-1 E2 expression in HeLa cells from a recombinant SV40 leads to a G₁ cell cycle arrest (Hwang et al. (1993) *J. Virol.*, 67:3720-3729). The HeLa-*tsE2* cell line was characterized for HPV-18 RNA levels. At the permissive temperature, the level of E6/E7 message was at least three- to fourfold lower than in HeLa-*tsE2* cells at 38°C. There was a temperature-dependent decrease of 1.5-fold in the HeLa-V cell line at 32°C compared with HeLa-V cells grown at 38°C.

15

(vi) *Effects of E2 expression on cellular proteins in HeLa cells.*

The E2-dependent loss of E6/E7 message and growth arrest may be mediated through cellular targets of the E6 and E7 proteins. Since HPV-18 E6 promotes the degradation of p53, the decrease in expression of the endogenous viral mRNA would be predicted to result in lower levels of E6 and increased levels of p53. The levels of p53 were examined in the HeLa-*tsE2* cell line following a shift to the permissive temperature. Immunoblot analysis of total cell extracts demonstrated a 20-fold increase in the level of p53 in HeLa-*tsE2* cells shifted to 32°C compared with normal HeLa cells or HeLa-*tsE2* cells grown at 38°C. A decrease in p53 levels was noted in HeLa-V cells at 32°C. The reason for this lowered level of p53 is not known, but this finding suggests that the destabilizing effect of E6 might be even greater than the 20-fold change in the HeLa-*tsE2* cell line.

Induction of the cyclin-dependent kinase inhibitor p21/WAF1/Cip1/Sdi1 occurs in response to increased levels of p53 (Eldiery et al. (1993) *Cell*, 75:817-825). An increased level of p21 protein was observed in the HeLa-*tsE2* cells after a shift to the permissive temperature (32°C). Notably, in the HeLa-V cell line at 32°C, which had very low levels of p53, no p21 was detected.

To examine whether the increased levels of p21 in the HeLa-*tsE2* cell line at the permissive temperature resulted in an inhibition of the cyclin-dependent kinases, cell lysates were prepared and immunoprecipitated with cyclin-specific antibodies, and kinase assays were performed with histone H1 as a substrate (Matsushime et al. (1994)

Mol. Cell Biol., 14:2066-2076). Cyclin E is associated with Cdk2 and is active in late G₁ (Dulic et al. (1993) *Proc. Natl. Acad. Sci. USA*, 90:11034-11038; Koff et al. 1992) *Science*, 257:1689-1694). Inhibition of the cyclin E-associated kinase activity was observed in the HeLa-*tsE2* cells at the permissive temperature. The cyclin E-associated kinase was active in the HeLa-*tsE2* cells at 38°C and in the HeLa-V cells at both 32 and 38°C. Cyclin A-associated kinase activity was also inhibited in the HeLa-*tsE2* cells at the permissive temperature but not at the nonpermissive temperature or in the HeLa-V cell line.

An immunoblot analysis of pRB was performed on extracts from the HeLa-*tsE2* and HeLa-V cell lines grown at 32 or 38°C. Both the hyperphosphorylated and hypophosphorylated forms of pRB were detected in each case; however, the pRB forms in the HeLa-*tsE2* cell line shifted to 32°C were predominantly hypophosphorylated. There was little difference in the phosphorylation state of pRB in the HeLa-*tsE2* cells grown at 38°C and pRB in the HeLa-V control cell line at either temperature.

Discussion

The E2 transactivation and DNA binding/dimerization domains were each necessary for E2-mediated growth suppression of HeLa cells. E2_{>220-309}, which is defective in the replication function but retains the ability to transactivate an E2-responsive plasmid (Winokur and McBride (1992) *EMBO J.*, 11:4111-4118), was able to suppress growth of HeLa cells, indicating that the growth-suppressive properties of E2 can be unlinked from its DNA replication properties. The fusion of two other transactivation domains to the E2 DNA binding domain did not suppress the growth of HeLa cells, even though each of these chimeric proteins could function as E2-dependent transactivators in these cells. Therefore, some characteristic of the E2 transactivation domain is specific for cell growth suppression and is not shared with other transactivation domains. While not wishing to be bound by any particular theory, it is possible that the E2 transactivation domain recruits specific cellular factors to the promoter which may play a role in the transcriptional repression and growth suppression activities of E2.

The dependence of a functional transactivation domain may be in part due to its ability to relieve nucleosome-mediated repression. The E2 transactivation domain has been shown to counteract the nucleosome repression of DNA replication (Li et al. (1994) *PNAS*, 91:7051-7055). The transcriptional repression properties of the E2-TR have been characterized on transiently transfected naked DNA. This, however, cannot be the only explanation for E2-mediated growth suppression, since the VP16

transactivation domain also has the ability to relieve nucleosome repression, but in our experiments, expression of the chimeric protein does not result in growth suppression.

The growth-suppressive effect of E2 was demonstrated in other HPV-positive cell lines but not in HPV-negative cell lines by the cotransfection assay used in this study. This result contrasts with the finding of Hwang et al. (Hwang et al. (1993) *J. Virol.*, 67:3720-3729), who observed a growth-suppressive effect with BPV-1 E2-TA in the HPV-negative cervical carcinoma cell line HT-3. One explanation for the difference between these two studies may involve the different assays used and different levels of expression of E2 achieved in the two experimental approaches. Our studies were done with DNA transfection, whereas the studies by Hwang et al. used the recombinant SV40 virus PAVA-E2, which resulted in much higher levels of protein expression. We have not been able to detect E2 protein by immunoblot analysis in HeLa cell lines transfected with the E2 plasmid, even though we can readily detect functional levels of E2 in transactivation of transcriptional repression assays. However, E2 is readily detectable by immunoblotting HeLa cells infected with the PAVA-E2 virus (Hwang et al. (1993) *J. Virol.*, 67:3720-3729), indicating a significantly higher level of expression. The two studies also differed in the experimental conditions to assess the biologic effects of E2 expression. Hwang et al. examined the transient effects of E2 by analyzing the cells 2 days postinfection. In the colony growth suppression assay used in our study, selection was maintained for 2 weeks, at which time the number of drug-resistant colonies was counted. Thus, the different effects of E2 observed on the growth of the HPV-negative HT-3 cell line may be due to differences in the assays, to the levels of E2 expression achieved by the different vectors, or to both. Nevertheless, it is the present findings which now provide the motivation to use E2 proteins to treat PV infected or transformed cells.

Repression of HPV-18 E6/E7 transcription was observed in the HeLa-*ts*E2 cells after a shift to the permissive temperature, resulting in a growth arrest. Our data indicate that in HeLa cells, the mechanism of E2-mediated growth arrest involves a decrease in E6 and E7 mRNA and reactivation of the p53/p21 and pRB pathways. The fact that the p21/Cdk inhibitory pathway is still intact and can be activated by disruption of E6 function suggests that therapeutic strategies targeted at interfering with E6 function could be effective in inhibition of cellular proliferation in HPV-positive cancers. We also observe an accumulation of hypophosphorylated pRB, which may be a consequence of a p53-mediated G₁ growth arrest (Slebos et al. (1994) *Proc. Natl. Acad. Sci. USA*, 91:5320-5324). Cells blocked in G₁ prior to activation of the cyclin D/Cdk complexes do not phosphorylate pRB. The shift in the phosphorylation state of RB to

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its hypophosphorylated form suggests that the cell cycle block occurs prior to the point of pRB phosphorylation. Our studies are in agreement with the observation that expression of antisense E6/E7 in C4-1, an HPV-18-derived cell line, was able to inhibit the growth rate of these cells (von Knebel-Doeberitz et al. (1988) *Cancer Res.*, 48 :3780-3785; von Knebel-Doeberitz et al. (1992) *Int. J. Cancer*, 51:831-834).

The studies presented here indicate that the transactivation domain of E2 is involved in the growth suppression and that the characteristic of the E2 transactivation domain in this suppression is not a general property shared with the VP16 and Spi transactivation domains.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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(2) INFORMATION FOR SEQ ID NO:1:

- 55 (i) SEQUENCE CHARACTERISTICS:

- 48 -

(A) LENGTH: 1095 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

10

(A) NAME/KEY: CDS

(B) LOCATION: 1..1095

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15	ATG GAG ACT CTT TGC CAA CGT TTA AAT GTG TGT CAG GAC AAA ATA CTA Met Glu Thr Leu Cys Gln Arg Leu Asn Val Cys Gln Asp Lys Ile Leu	48
	1 5 10 15	
20	ACA CAT TAT GAA AAT GAT AGT ACA GAC CTA CGT GAC CAT ATA GAC TAT Thr His Tyr Glu Asn Asp Ser Thr Asp Leu Arg Asp His Ile Asp Tyr	96
	20 25 30	
25	TGG AAA CAC ATG CGC CTA GAA TGT GCT ATT TAT TAC AAG GCC AGA GAA Trp Lys His Met Arg Leu Glu Cys Ala Ile Tyr Tyr Lys Ala Arg Glu	144
	35 40 45	
30	ATG GGA TTT AAA CAT ATT AAC CAC CAA GTG GTG CCA ACA CTG GCT GTA Met Gly Phe Lys His Ile Asn His Gln Val Val Pro Thr Leu Ala Val	192
	50 55 60	
35	TCA AAG AAT AAA GCA TTA CAA GCA ATT GAA CTG CAA CTA ACG TTA GAA Ser Lys Asn Lys Ala Leu Gln Ala Ile Glu Leu Gln Leu Thr Leu Glu	240
	65 70 75 80	
40	ACA ATA TAT AAC TCA CAA TAT AGT AAT GAA AAG TGG ACA TTA CAA GAC Thr Ile Tyr Asn Ser Gln Tyr Ser Asn Glu Lys Trp Thr Leu Gln Asp	288
	85 90 95	
45	GTT AGC CTT GAA GTG TAT TTA ACT GCA CCA ACA GGA TGT ATA AAA AAA Val Ser Leu Glu Val Tyr Leu Thr Ala Pro Thr Gly Cys Ile Lys Lys	336
	100 105 110	
50	CAT GGA TAT ACA GTG GAA GTG CAG TTT GAT GGA GAC ATA TGC AAT ACA His Gly Tyr Thr Val Glu Val Gln Phe Asp Gly Asp Ile Cys Asn Thr	384
	115 120 125	
55	ATG CAT TAT ACA AAC TGG ACA CAT ATA TAT ATT TGT GAA GAA GCA TCA Met His Tyr Thr Asn Trp Thr His Ile Tyr Ile Cys Glu Glu Ala Ser	432
	130 135 140	
60	GTA ACT GTG GTA GAG GGT CAA GTT GAC TAT TAT GGT TTA TAT TAT GTT Val Thr Val Val Glu Gly Gln Val Asp Tyr Tyr Gly Leu Tyr Tyr Val	480
	145 150 155 160	
65	CAT GAA GGA ATA CGA ACA TAT TTT GTG CAG TTT AAA GAT GAT GCA GAA His Glu Gly Ile Arg Thr Tyr Phe Val Gln Phe Lys Asp Asp Ala Glu	528

- 49 -

	165	170	175	
5	AAA TAT AGT AAA AAT AAA GTA TGG GAA GTT CAT GCG GGT GGT CAG GTA Lys Tyr Ser Lys Asn Lys Val Trp Glu Val His Ala Gly Gly Gln Val 180 185 190	576		
10	ATA TTA TGT CCT ACA TCT GTG TTT AGC AGC AAC GAA GTA TCC TCT CCT Ile Leu Cys Pro Thr Ser Val Phe Ser Ser Asn Glu Val Ser Ser Pro 195 200 205	624		
	GAA ATT ATT AGG CAG CAC TTG GCC AAC CAC CCC GCC GCG ACC CAT ACC Glu Ile Ile Arg Gln His Leu Ala Asn His Pro Ala Ala Thr His Thr 210 215 220	672		
15	AAA GCC GTC GCC TTG GGC ACC GAA GAA ACA CAG ACG ACT ATC CAG CGA Lys Ala Val Ala Leu Gly Thr Glu Glu Thr Gln Thr Thr Ile Gln Arg 225 230 235 240	720		
20	CCA AGA TCA GAG CCA GAC ACC GGA AAC CCC TGC CAC ACC ACT AAG TTG Pro Arg Ser Glu Pro Asp Thr Gly Asn Pro Cys His Thr Thr Lys Leu 245 250 255	768		
25	TTG CAC AGA GAC TCA GTG GAC AGT GCT CCA ATC CTC ACT GCA TTT AAC Leu His Arg Asp Ser Val Asp Ser Ala Pro Ile Leu Thr Ala Phe Asn 260 265 270	816		
30	AGC TCA CAC AAA GGA CGG ATT AAC TGT AAT AGT AAC ACT ACA CCC ATA Ser Ser His Lys Gly Arg Ile Asn Cys Asn Ser Asn Thr Thr Pro Ile 275 280 285	864		
	GTA CAT TTA AAA GGT GAT GCT AAT ACT TTA AAA TGT TTA AGA TAT AGA Val His Leu Lys Gly Asp Ala Asn Thr Leu Lys Cys Leu Arg Tyr Arg 290 295 300	912		
35	TTT AAA AAG CAT TGT ACA TTG TAT ACT GCA GTG TCG TCT ACA TGG CAT Phe Lys Lys His Cys Thr Leu Tyr Thr Ala Val Ser Ser Thr Trp His 305 310 315 320	960		
40	TGG ACA GGA CAT AAT GTA AAA CAT AAA AGT GCA ATT GTT ACA CTT ACA Trp Thr Gly His Asn Val Lys His Lys Ser Ala Ile Val Thr Leu Thr 325 330 335	1008		
45	TAT GAT AGT GAA TGG CAA CGT GAC CAA TTT TTG TCT CAA GTT AAA ATA Tyr Asp Ser Glu Trp Gln Arg Asp Gln Phe Leu Ser Gln Val Lys Ile 340 345 350	1056		
50	CCA AAA ACT ATT ACA GTG TCT ACT GGA TTT ATG TCT ATA Pro Lys Thr Ile Thr Val Ser Thr Gly Phe Met Ser Ile 355 360 365	1095		

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

55 (A) LENGTH: 365 amino acids

- 50 -

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Thr Leu Cys Gln Arg Leu Asn Val Cys Gln Asp Lys Ile Leu
 1 5 10 15
 Thr His Tyr Glu Asn Asp Ser Thr Asp Leu Arg Asp His Ile Asp Tyr
 20 25 30
 Trp Lys His Met Arg Leu Glu Cys Ala Ile Tyr Tyr Lys Ala Arg Glu
 35 40 45
 Met Gly Phe Lys His Ile Asn His Gln Val Val Pro Thr Leu Ala Val
 50 55 60
 Ser Lys Asn Lys Ala Leu Gln Ala Ile Glu Leu Gln Leu Thr Leu Glu
 65 70 75 80
 Thr Ile Tyr Asn Ser Gln Tyr Ser Asn Glu Lys Trp Thr Leu Gln Asp
 85 90 95
 Val Ser Leu Glu Val Tyr Leu Thr Ala Pro Thr Gly Cys Ile Lys Lys
 100 105 110
 His Gly Tyr Thr Val Glu Val Gln Phe Asp Gly Asp Ile Cys Asn Thr
 115 120 125
 Met His Tyr Thr Asn Trp Thr His Ile Tyr Ile Cys Glu Glu Ala Ser
 130 135 140
 Val Thr Val Val Glu Gly Gln Val Asp Tyr Tyr Gly Leu Tyr Tyr Val
 145 150 155 160
 His Glu Gly Ile Arg Thr Tyr Phe Val Gln Phe Lys Asp Asp Ala Glu
 165 170 175
 Lys Tyr Ser Lys Asn Lys Val Trp Glu Val His Ala Gly Gly Gln Val
 180 185 190
 Ile Leu Cys Pro Thr Ser Val Phe Ser Ser Asn Glu Val Ser Ser Pro
 195 200 205
 Glu Ile Ile Arg Gln His Leu Ala Asn His Pro Ala Ala Thr His Thr
 210 215 220
 Lys Ala Val Ala Leu Gly Thr Glu Glu Thr Gln Thr Thr Ile Gln Arg
 225 230 235 240
 Pro Arg Ser Glu Pro Asp Thr Gly Asn Pro Cys His Thr Thr Lys Leu
 245 250 255

55

- 51 -

Leu His Arg Asp Ser Val Asp Ser Ala Pro Ile Leu Thr Ala Phe Asn
 260 265 270
 5 Ser Ser His Lys Gly Arg Ile Asn Cys Asn Ser Asn Thr Thr Pro Ile
 275 280 285
 Val His Leu Lys Gly Asp Ala Asn Thr Leu Lys Cys Leu Arg Tyr Arg
 290 295 300
 10 Phe Lys Lys His Cys Thr Leu Tyr Thr Ala Val Ser Ser Thr Trp His
 305 310 315 320
 Trp Thr Gly His Asn Val Lys His Lys Ser Ala Ile Val Thr Leu Thr
 325 330 335
 15 Tyr Asp Ser Glu Trp Gln Arg Asp Gln Phe Leu Ser Gln Val Lys Ile
 340 345 350
 Pro Lys Thr Ile Thr Val Ser Thr Gly Phe Met Ser Ile
 20 355 360 365

(4) INFORMATION FOR SEQ ID NO:3:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1095 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1095

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

40	ATG CAG ACA CCG AAG GAA ACC CTT TCG GAA CGT TTA AGT TGC GTG CAG	48
	Met Gln Thr Pro Lys Glu Thr Leu Ser Glu Arg Leu Ser Cys Val Gln	
	1 5 10 15	
45	GAC AAA ATC ATA GAC CAC TAT GAA AAT GAC AGT AAA GAC ATA GAC AGC	96
	Asp Lys Ile Ile Asp His Tyr Glu Asn Asp Ser Lys Asp Ile Asp Ser	
	20 25 30	
50	CAA ATA CAG TAT TGG CAA CTA ATA CGT TGG GAA AAT GCA ATA TTC TTT	144
	Gln Ile Gln Tyr Trp Gln Leu Ile Arg Trp Glu Asn Ala Ile Phe Phe	
	35 40 45	
55	GCA GCA AGG GAA CAT GGC ATA CAG ACA TTA AAC CAC CAG GTG GTG CCA	192
	Ala Ala Arg Glu His Gly Ile Gln Thr Leu Asn His Gln Val Val Pro	
	50 55 60	
55	GCC TAT AAC ATT TCA AAA AGT AAA GCA CAT AAA GCT ATT GAA CTG CAA	240
	Ala Tyr Asn Ile Ser Lys Ser Lys Ala His Lys Ala Ile Glu Leu Gln	

- 52 -

	65	70	75	80	
5	ATG GCC CTA CAA GGC CTT GCA CAA AGT CGA TAC AAA ACC GAG GAT TGG Met Ala Leu Gln Gly Leu Ala Gln Ser Arg Tyr Lys Thr Glu Asp Trp	85	90	95	288
10	ACA CTG CAA GAC ACA TGC GAG GAA CTA TGG AAT ACA GAA CCT ACT CAC Thr Leu Gln Asp Thr Cys Glu Glu Leu Trp Asn Thr Glu Pro Thr His	100	105	110	336
15	TGC TTT AAA AAA GGT GGC CAA ACA GTA CAA GTA TAT TTT GAT GGC AAC Cys Phe Lys Lys Gly Gly Gln Thr Val Gln Val Tyr Phe Asp Gly Asn	115	120	125	384
20	AAA GAC AAT TGT ATG ACC TAT GTA GCA TGG GAC AGT GTG TAT TAT ATG Lys Asp Asn Cys Met Thr Tyr Val Ala Trp Asp Ser Val Tyr Tyr Met	130	135	140	432
25	ACT GAT GCA GGA ACA TGG GAC AAA ACC GCT ACC TGT GTA AGT CAC AGG Thr Asp Ala Gly Thr Trp Asp Lys Thr Ala Thr Cys Val Ser His Arg	145	150	155	480
30	GGA TTG TAT TAT GTA AAG GAA GGG TAC AAC ACG TTT TAT ATA GAA TTT Gly Leu Tyr Tyr Val Lys Glu Gly Tyr Asn Thr Phe Tyr Ile Glu Phe	165	170	175	528
35	AAA AGT GAA TGT GAA AAA TAT GGG AAC ACA GGT ACG TGG GAA GTA CAT Lys Ser Glu Cys Glu Lys Tyr Gly Asn Thr Gly Thr Trp Glu Val His	180	185	190	576
40	TTT GGG AAT AAT GTA ATT GAT TGT AAT GAC TCT ATG TGC AGT ACC AGT Phe Gly Asn Asn Val Ile Asp Cys Asn Asp Ser Met Cys Ser Thr Ser	195	200	205	624
45	GAC GAC ACG GTA TCC GCT ACT CAG CTT GTT AAA CAG CTA CAG CAC ACC Asp Asp Thr Val Ser Ala Thr Gln Leu Val Lys Gln Leu Gln His Thr	210	215	220	672
50	CCC TCA CCG TAT TCC AGC ACC GTG TCC GTG GGC ACC GCA AAG ACC TAC Pro Ser Pro Tyr Ser Ser Thr Val Ser Val Gly Thr Ala Lys Thr Tyr	225	230	235	720
55	GGC CAG ACG TCG GCT GCT ACA CGA CCT GGA CAC TGT GGA CTC GCG GAG Gly Gln Thr Ser Ala Ala Thr Arg Pro Gly His Cys Gly Leu Ala Glu	245	250	255	768
60	AAG CAG CAT TGT GGA CCT GTC AAC CCA CTT CTC GGT GCA GCT ACA CCT Lys Gln His Cys Gly Pro Val Asn Pro Leu Leu Gly Ala Ala Thr Pro	260	265	270	816
65	ACA GGC AAC AAC AAA AGA CGG AAA CTC TGT AGT GGT AAC ACT ACG CCT Thr Gly Asn Asn Lys Arg Arg Lys Leu Cys Ser Gly Asn Thr Thr Pro	275	280	285	864
70	ATA ATA CAT TTA AAA GGT GAC AGA AAC AGT TTA AAA TGT TTA CGG TAC				912

- 53 -

Ile Ile His Leu Lys Gly Asp Arg Asn Ser Leu Lys Cys Leu Arg Tyr
 290 295 300

5 AGA TTG CGA AAA CAT AGC GAC CAC TAT AGA GAT ATA TCA TCC ACC TGG 960
 Arg Leu Arg Lys His Ser Asp His Tyr Arg Asp Ile Ser Ser Thr Trp
 305 310 315 320

10 CAT TGG ACA GGT GCA GGC AAT GAA AAA ACA GGA ATA CTG ACT GTA ACA 1008
 His Trp Thr Gly Ala Gly Asn Glu Lys Thr Gly Ile Leu Thr Val Thr
 325 330 335

15 TAC CAT AGT GAA ACA CAA AGA ACA AAA TTT TTA AAT ACT GTT GCA ATT 1056
 Tyr His Ser Glu Thr Gln Arg Thr Lys Phe Leu Asn Thr Val Ala Ile
 340 345 350

CCA GAT AGT GTA CAA ATA TTG GTG GGA TAC ATG ACA ATG 1095
 Pro Asp Ser Val Gln Ile Leu Val Gly Tyr Met Thr Met
 355 360 365

20

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 365 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: protein

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gln Thr Pro Lys Glu Thr Leu Ser Glu Arg Leu Ser Cys Val Gln
 1 5 10 15

35 Asp Lys Ile Ile Asp His Tyr Glu Asn Asp Ser Lys Asp Ile Asp Ser
 20 25 30

Gln Ile Gln Tyr Trp Gln Leu Ile Arg Trp Glu Asn Ala Ile Phe Phe
 35 40 45

40 Ala Ala Arg Glu His Gly Ile Gln Thr Leu Asn His Gln Val Val Pro
 50 55 60

45 Ala Tyr Asn Ile Ser Lys Ser Lys Ala His Lys Ala Ile Glu Leu Gln
 65 70 75 80

Met Ala Leu Gln Gly Leu Ala Gln Ser Arg Tyr Lys Thr Glu Asp Trp
 85 90 95

50 Thr Leu Gln Asp Thr Cys Glu Glu Leu Trp Asn Thr Glu Pro Thr His
 100 105 110

Cys Phe Lys Lys Gly Gly Gln Thr Val Gln Val Tyr Phe Asp Gly Asn
 115 120 125

55

- 54 -

Lys Asp Asn Cys Met Thr Tyr Val Ala Trp Asp Ser Val Tyr Tyr Met
 130 135 140

5 Thr Asp Ala Gly Thr Trp Asp Lys Thr Ala Thr Cys Val Ser His Arg
 145 150 155 160

Gly Leu Tyr Tyr Val Lys Glu Gly Tyr Asn Thr Phe Tyr Ile Glu Phe
 165 170 175

10 Lys Ser Glu Cys Glu Lys Tyr Gly Asn Thr Gly Thr Trp Glu Val His
 180 185 190

Phe Gly Asn Asn Val Ile Asp Cys Asn Asp Ser Met Cys Ser Thr Ser
 195 200 205

15 Asp Asp Thr Val Ser Ala Thr Gln Leu Val Lys Gln Leu Gln His Thr
 210 215 220

20 Pro Ser Pro Tyr Ser Ser Thr Val Ser Val Gly Thr Ala Lys Thr Tyr
 225 230 235 240

Gly Gln Thr Ser Ala Ala Thr Arg Pro Gly His Cys Gly Leu Ala Glu
 245 250 255

25 Lys Gln His Cys Gly Pro Val Asn Pro Leu Leu Gly Ala Ala Thr Pro
 260 265 270

Thr Gly Asn Asn Lys Arg Arg Lys Leu Cys Ser Gly Asn Thr Thr Pro
 275 280 285

30 Ile Ile His Leu Lys Gly Asp Arg Asn Ser Leu Lys Cys Leu Arg Tyr
 290 295 300

Arg Leu Arg Lys His Ser Asp His Tyr Arg Asp Ile Ser Ser Thr Trp
 305 310 315 320

His Trp Thr Gly Ala Gly Asn Glu Lys Thr Gly Ile Leu Thr Val Thr
 325 330 335

40 Tyr His Ser Glu Thr Gln Arg Thr Lys Phe Leu Asn Thr Val Ala Ile
 340 345 350

Pro Asp Ser Val Gln Ile Leu Val Gly Tyr Met Thr Met
 355 360 365

- 55 -

We claim:

1. A method of treating a papillomavirus-induced condition, comprising administering to a mammal infected with a papillomavirus a pharmaceutical preparation comprising a therapeutically effective amount of (i) an E2 polypeptide or (ii) a gene construct for expressing an E2 polypeptide, wherein the E2 polypeptide includes a DNA binding domain and a transcriptional activation domain, and the E2 polypeptide or gene construct is formulated in the pharmaceutical preparation for delivery into papillomavirus-infected cells of the animal.
2. The method of claim 1, wherein the mammal is a livestock, zoo animal or pet.
3. The method of claim 1, wherein the mammal is a human.
4. The method of claim 2, wherein the human is infected with a human papillomavirus (HPV).
5. The method of claim 4, wherein the HPV is a high risk HPV.
6. The method of claim 5, wherein the high risk HPV is selected from the group consisting of HPV-16, HPV-18, HPV-31 and HPV-33.
7. The method of claim 4, wherein the HPV is a low risk HPV.
8. The method of claim 1, wherein the DNA binding domain and transcriptional activation domain of the E2 polypeptide have amino acid sequences corresponding to an E2 protein from an HPV.
9. The method of claim 8, wherein the DNA binding domain and a transcriptional activation domain of the E2 polypeptide have amino acid sequences corresponding to an E2 protein from a high risk HPV.
10. The method of claim 9, wherein the DNA binding domain and a transcriptional activation domain of the E2 polypeptide have amino acid sequences

- 56 -

corresponding to an E2 protein from the group consisting of HPV-16, HPV-18, HPV-31 and HPV-33.

11. The method of claim 1, wherein the papillomavirus-induced
5 condition being treated includes growth of papillomavirus-transformed cells.

12. The method of claim 1, wherein the papillomavirus-induced condition being treated includes growth of warts.

10 13. A method of preventing or reversing papillomavirus-induced conditions associated with human papillomavirus (HPV) infection, comprising administering to an HPV-infected patient a pharmaceutical preparation comprising a therapeutically effective amount of an E2_{ad/db} polypeptide or a gene construct for expressing the E2_{ad/db} polypeptide, formulated in the pharmaceutical preparation
15 for delivery into HPV-infected cells of the animal, wherein the papillomavirus-induced condition is selected from the group consisting of the formation of warts and the transformation of HPV-infected cells.

14. The method of claim 13, wherein the papillomavirus-induced
20 condition being treated is selected from the group consisting of Plantar warts (verruca plantaris), common warts (verruca plana), Butcher's common warts, flat warts, genital warts (condyloma acuminatum), and epidermodysplasia verruciformis.

25 15. The method of claim 13, wherein the papillomavirus-induced condition being treated is selected from the group consisting of laryngeal papilloma and cervical carcinoma.

16. A method of treating a papillomavirus-induced condition, comprising
30 administering to a subject infected with a high risk human papillomavirus (HPV) a pharmaceutical preparation comprising an E2 polypeptide, wherein the E2 polypeptide includes a DNA binding domain and a transcriptional activation domain, and the E2 polypeptide is formulated in the pharmaceutical preparation for delivery into PV-infected cells of the animal.

35

- 57 -

17. The method of claim 16, wherein the high risk HPV is selected from the group consisting of HPV-16, HPV-18, HPV-31 and HPV-33.
18. The method of claim 16, wherein the DNA binding domain and transcriptional activation domain of the E2 polypeptide are chemically cross-linked.
19. The method of claim 16, wherein the DNA binding domain and a transcriptional activation domain of the E2 polypeptide are portions of one contiguous polypeptide chain.
20. The method of claim 16, wherein the DNA binding domain and transcriptional activation domain of the E2 polypeptide have amino acid sequences corresponding to an E2 protein from an HPV.
21. The method of claim 20, wherein the DNA binding domain and transcriptional activation domain of the E2 polypeptide have amino acid sequences corresponding to an E2 protein from a high risk HPV.
22. The method of claim 20, wherein E2 polypeptide has an amino acid sequence corresponding to an E2 protein selected from the group consisting of SEQ ID No. 2 and SEQ ID No. 4.
23. The method of claim 16, wherein the papillomavirus-induced condition being treated includes growth of HPV-transformed cells.
24. The method of claim 16, wherein the papillomavirus-induced condition being treated includes growth of warts.
25. The method of claim 16, wherein the E2 polypeptide is formulated in liposomes.
26. A method of treating a papillomavirus-induced condition, comprising administering to a subject infected with a high risk human papillomavirus (HPV) a pharmaceutical preparation comprising a gene construct for expressing an E2 polypeptide including a DNA binding domain and a transcriptional activation domain,

- 58 -

wherein the gene construct is formulated in the pharmaceutical preparation for delivery into PV-infected cells of the animal.

27. The method of claim 26, wherein the high risk HPV is selected from
5 the group consisting of HPV-16, HPV-18, HPV-31 and HPV-33.

28. The method of claim 26, wherein the DNA binding domain and
transcriptional activation domain of the E2 polypeptide are interrupted by a hinge
polypeptide sequence.
10

29. The method of claim 26, wherein the DNA binding domain and a
transcriptional activation domain of the E2 polypeptide have amino acid sequences
corresponding to an E2 protein from a human papillomavirus (HPV).

30. The method of claim 29, wherein the DNA binding domain and a
transcriptional activation domain of the E2 polypeptide have amino acid sequences
corresponding to an E2 protein from a high risk HPV.
15

31. The method of claim 30, wherein E2 polypeptide has an amino acid
20 sequence corresponding to an E2 protein selected from the group consisting of SEQ
ID No. 2 and SEQ ID No. 4.

32. The method of claim 26, wherein the gene construct is a viral vector.

33. The method of claim 26, wherein the gene construct is formulated in
liposomes.
25

34. The method of claim 26, wherein the papillomavirus-induced
condition being treated includes growth of HPV-transformed cells.
30

35. The method of claim 26, wherein the papillomavirus-induced condition
being treated includes growth of warts.

36. A pharmaceutical preparation comprising a therapeutically effective
35 amount of a recombinant transfection system for ameliorating a papillomavirus-induced
condition in a subject, comprising

- 59 -

(i) a gene construct including the nucleic acid encoding an E2 polypeptide and operably linked to a transcriptional regulatory sequence for causing expression of the E2 polypeptide in eukaryotic cells, the E2 polypeptide including a DNA binding domain and a transcriptional activation domain; and

5 (ii) a gene delivery composition for delivering said gene construct to a papillomavirus infected cells of the subject and causing the cell to be transfected with said gene construct.

37. The preparation of claim 36, wherein the gene delivery composition is
10 selected from a group consisting of a recombinant viral particle, a liposome, and a polycationic nucleic acid binding agent.

38. The preparation of claim 36, wherein the DNA binding domain and a
15 transcriptional activation domain of the E2 polypeptide have amino acid sequences corresponding to an E2 protein from a human papillomavirus (HPV).

39. The preparation of claim 38, wherein the DNA binding domain and a
transcriptional activation domain of the E2 polypeptide have amino acid sequences
20 corresponding to an E2 protein from a high risk HPV.

40. The preparation of claim 39, wherein E2 polypeptide has an amino
acid sequence corresponding to an E2 protein selected from the group consisting of
SEQ ID No. 2 and SEQ ID No. 4.

25 41. A pharmaceutical preparation comprising a therapeutically effective amount of an E2 polypeptide, the E2 polypeptide including a DNA binding domain and a transcriptional activation domain, and formulated in the pharmaceutical preparation for delivery into PV-infected cells of an animal.

30 42. The preparation of claim 41, wherein the DNA binding domain and transcriptional activation domain of the E2 polypeptide are chemically cross-linked.

43. The preparation of claim 41, wherein the DNA binding domain and
transcriptional activation domain of the E2 polypeptide are portions of one
35 contiguous polypeptide chain.

- 60 -

44. The preparation of claim 41, wherein the DNA binding domain and a transcriptional activation domain of the E2 polypeptide have amino acid sequences corresponding to an E2 protein from a human papillomavirus (HPV).
- 5 45. The preparation of claim 44, wherein the DNA binding domain and a transcriptional activation domain of the E2 polypeptide have amino acid sequences corresponding to an E2 protein from a high risk HPV.
- 10 46. The preparation of claim 45, wherein E2 polypeptide has an amino acid sequence corresponding to an E2 protein selected from the group consisting of SEQ ID No. 2 and SEQ ID No. 4.
47. The preparation of claim 41, wherein the E2 polypeptide is formulated in liposomes.

Figure 1

G418 Resistant Colonies

Exp. 1 Exp. 2 Exp. 3

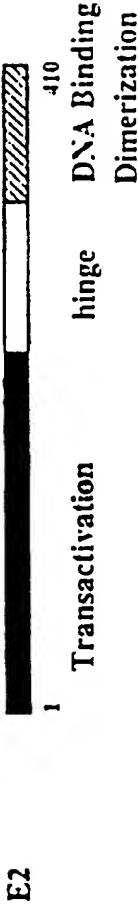


E2-TA



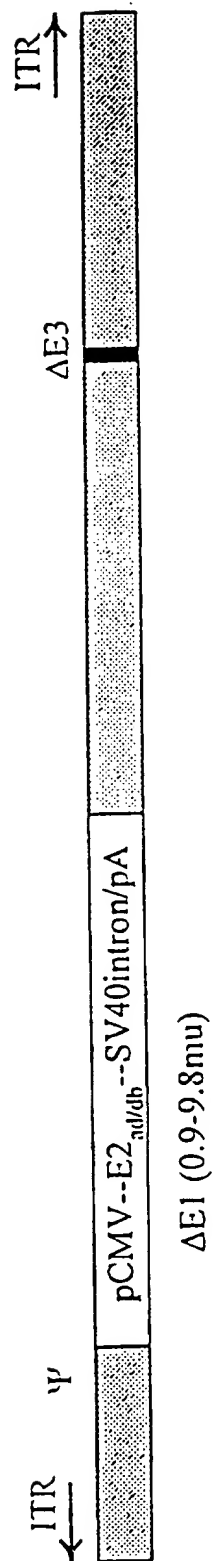
E2-TR

Vector alone



E2

2/4

Figure 2

3/4

Figure 3A



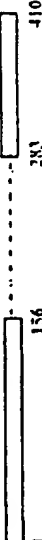
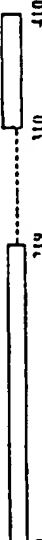

	E2 Properties		G418 resistant colonies	
	DNA replication	Transcriptional trans-activation	Exp. 1	Exp. 2
E2 $\Delta 1-15$		-	38	46
E2 1-218		+	82	47
E2 $\Delta 157-282$		-	69	52
E2 $\Delta 220-349$		+	0	0
E2TA		+	0	0
Vector Alone	NA	NA	42	55

Figure 3B

	E2 Properties			G418 resistant colonies	
	transcriptional transactivation	dimerization	DNA binding	Exp. 1	Exp. 2
spi-E2	+	+	+	78	80
VP16-E2	+	+	+	92	114
E2-TA	+	+	+	0	0
E2 (I 331 R)	-	-	-	45	73
E2 (R 344 K)	-	+	-	51	58

INTERNATIONAL SEARCH REPORT

Intern. al Application No

PCT/US 97/11815

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 A61K38/16 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92 12728 A (BIOGEN INC ;NEW ENGLAND MEDICAL CENTER HOR (US)) 6 August 1992 see page 1, line 4 - line 22 see page 7, line 25 - page 8, line 33 see page 27, line 10 - line 25 ---	1-47
A	MCBRIDE ET AL: "THE PAPILLOMAVIRUS E2 REGULATORY PROTEINS" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 28, 5 October 1991, pages 18411-18414, XP002045852 cited in the application see the whole document --- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier document but published on or after the international filing date
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 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
 "&" document member of the same patent family

Date of the actual completion of the international search

5 November 1997

Date of mailing of the international search report

25. 11. 97

Name and mailing address of the ISA

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 Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

INTERNATIONAL SEARCH REPORT

Intern of Application No

PCT/US 97/11815

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>GIRI ET AL: "STRUCTURAL AND MUTATIONAL ANALYSIS OF E2 TRANS-ACTIVATING PROTEINS OF PAPILLOMAVIRUS REVEALS THREE DISTINCT FUNCTIONAL DOMAINS" THE EMBO JOURNAL, vol. 7, no. 9, 1988, pages 2823-2829, XP002045853 cited in the application see the whole document</p> <p style="text-align: center;">---</p>	
A	<p>BOUVARD ET AL: "CHARACTERIZATION OF THE HUMAN PAPILLOMAVIRUS E2 PROTEIN: EVIDENCE OF TRANS-ACTIVATION AND TRANS-REPRESSION IN CERVICAL KERATINOCYTES" THE EMBO JOURNAL, vol. 13, no. 22, 1994, pages 5451-5459, XP002045854 see the whole document</p> <p style="text-align: center;">-----</p>	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/11815

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 1-35
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/11815

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9212728 A	06-08-92	US 5219990 A	15-06-93
		AU 669843 B	27-06-96
		AU 1242492 A	27-08-92
		CA 2099976 A	29-07-92
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		JP 6506344 T	21-07-94
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